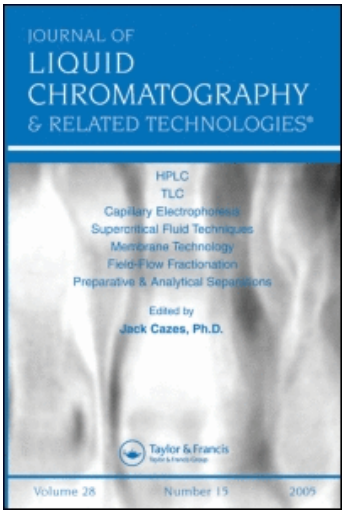

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A HPLC Screening Procedure for Sulfamethazine Residues in Pork Tissues

Arnost B. Vilim[a]; Lyse Larocque[a]; Agnes I. Macintosh[a]
[a] Drug Research Laboratories Health Protection Branch Health and Welfare Canada, Ottawa, Canada

To cite this Article Vilim, Arnost B. , Larocque, Lyse and Macintosh, Agnes I.(1980) 'A HPLC Screening Procedure for Sulfamethazine Residues in Pork Tissues', Journal of Liquid Chromatography & Related Technologies, 3: 11, 1725 — 1736
To link to this Article: DOI: 10.1080/01483918008064763
URL: http://dx.doi.org/10.1080/01483918008064763

JOURNAL OF LIQUID CHROMATOGRAPHY, 3(11), 1725-1736 (1980)

A HPLC SCREENING PROCEDURE FOR SULFAMETHAZINE RESIDUES IN PORK TISSUES

Arnost B. Vilim, Lyse Larocque and Agnes I. MacIntosh

Drug Research Laboratories
Health Protection Branch
Health and Welfare Canada
Ottawa, Canada, K1A 0L2

ABSTRACT

A sensitive and specific screening procedure is described for the quantitative detection of sulfamethazine residues in pork kidney, liver and muscle. Initial screening is by both the Bratton-Marshall reaction, and by high pressure liquid chromatography (HPLC); quantitation is by HPLC; identification is then confirmed by means of thin-layer chromatography (TLC) of the derivatized standards and the unknown from the Bratton-Marshall reaction. Only one extraction of a 50g sample is needed, one portion (10g tissue equivalent) is used for the colorimetric reaction and TLC confirmation, and another portion (25g tissue equivalent) is used for quantitative HPLC determination. Standard curves for sulfamethazine are constructed for each tissue at 50, 100, 200 and 500 ppb levels. The average mean recovery for all tissues at all levels is 78.2%. The method is verified by a 150 sample survey using 50 samples of each tissue from local supermarkets. Approximately 4% of the samples show contamination ranging from a level of 100 ppb to 3 ppm.

INTRODUCTION

It is common practice in modern swine rearing to use sulfonamide drugs for prevention and treatment of disease, as well as to promote growth. There is therefore a need to monitor human foods for drug residues resulting from both the growth promotion and treatment levels. Essential to the success of such monitoring is the availability of reliable methodology capable of determining significant levels of drugs and their metabolites. Due to the large number of

samples which must be surveyed for sulfamethazine it is of the utmost importance that the method be readily practicable. It has to measure reliably the compound(s) in question, should be rapid and reasonable in terms of required expertise and equipment; it must also exhibit sensitivity well below the 100 ppb level of sulfonamide residue (in edible pork tissues) set by U.S. federal regulations (1), and subscribed to by Canada.

Current analytical methods in use are based on the Bratton-Marshall reaction (2) and are modified for use in milk and tissues (3). Several TLC systems are described in the literature (4,5) and recently, HPLC (6) and gas-liquid chromatography (GLC) (7) procedures were reported.

The HPLC screening procedure described in this paper was designed primarily for sulfamethazine residues. However, it does separate 14 sulfonamides approved for use in swine in Canada and is specific in combination with TLC confirmation. Improvements are made in the extraction procedure, and since there are no derivatization steps considerable saving of time can be achieved with this method as compared to the GLC (7) procedure. Furthermore, the method does not require the tedious separations involved in TLC assays (4,5) and recoveries and separations are better than for the published HPLC procedure(6).

MATERIALS AND METHODS

Chemicals

Ethanol, chloroform, acetone, hexane, ethyl ether, methylene chloride, 1-butanol, and methanol were either reagent grade or "Distilled-in-Glass" solvents, obtained from Caledon Laboratories Ltd., Georgetown, Ontario L7G 4R9, or equivalent. Ammonium acetate, reagent grade, was purchased from Anachemia Chemicals Ltd., Montreal, Quebec H8S 4A7. Hydrochloric acid, reagent grade, was purchased from J.T. Baker Chemical Co., Phillipsburg, N.J. 08865. Ammonium sulfamate, ammonium hydroxide and N-(1-Naphthyl)-ethylenediamine di-

hydrochloride (NED) reagent grade, were obtained from Fisher Scientific Co., Fairlawn, N.J. 07410. Sodium nitrite, sodium hydroxide and tri-sodium citrate, reagent grade, were purchased from BDH Chemicals, Toronto, Ontario M8F 1K5. Glacial acetic acid, reagent grade, was obtained from Mallinckrodt Chemical Works Ltd., Toronto, Ontario. Celite 545 was purchased from Chromatographic Specialties Ltd., Brockville, Ontario K6V 5W1.

Apparatus

Samples were homogenized in a Waring Laboratory Blendor, Waring Products Division of Dynamics Corporation of America, New Hartford, Conn. Evaporation was carried out in a Buchler Flash-Evaporator, Buchler Instruments, Fort Lee, N.J. The pH was measured using a Corning 125 pH meter, Johns Scientific, Toronto, Ontario M4M 2G4. Vortex Jr. mixer, Scientific Industries Inc., Springfield, Mass. 01103 was used to mix samples for the Bratton-Marshall reaction. Eastman Chromagram Developing Apparatus 13259, Eastman Kodak Co., Rochester, N.Y. 14650, and silica gel plates with fluorescent indicator (No. 6060) were employed for TLC separations. Waters Associates Model ALC/GPC-204 liquid chromatograph, Waters Associates, Mississauga, Ontario L4V 1H3, equipped with twin channel Waters' Model 440 Absorbance Detector, two M 6000 A solvent delivery systems and Waters' Model U6K Universal Injectors was used. Reverse phase C-8, 10 µm analytical column (Brownlee Labs RP-10A, Santa Clara, Ca. 95050) and a column inlet filter with a 2 µm filter element (Model 7302, Rheodyne, Berkeley, Ca. 94710) were connected to the liquid chromatograph. In order to check the HPLC system for reproducibility, two columns of identical designation and from the same manufacturer but different lot number were used and called system 1 and system 2 in Tables 2 and 3. Quantitation data (peak area) were obtained on a Hewlett-Packard Model 3385 Reporting Integrator, Hewlett-Packard Canada Ltd., Ottawa, Ontario K2C 0P9.

Standard Solution

Weigh out 5.0 mg of sulfamethazine and dissolve in ethanol in a 100 ml volumetric flask. Dilute this solution 1/10 to give a final concentration of standard solution of 5 µg/ml.

Standard Curve

Spike 50 gm quantities of blank tissue with 0.5, 1.0, 2.0 and 5.0 ml of sulfamethazine standard solution (5 µg/ml) to give 50, 100, 200, and 500 ppb levels.

Sample Preparation

Place 50 gm of diced tissue (unknown sample or spiked standard) in a blending jar and homogenize with 50 ml ethanol at high speed for 1 minute, then add 150 ml ethanol and homogenize at medium speed for 2 minutes. Filter the homogenate through a 5 mm thick layer of pre-washed celite (50 ml ethanol) in a 350 ml medium porosity sintered glass funnel. Rinse the blending jar and the funnel with another 100 ml ethanol. Transfer combined filtrates into a 1l round bottom flask and evaporate to dryness at 50°C on a rotary evaporator. (Care should be taken with some tissues, esp. liver, to prevent the sample from boiling over.) Dissolve the residue in 25.0 ml IN HCl and transfer into a 250 ml separatory funnel. Rinse the flask with 35 ml ethyl ether and transfer to funnel. Repeat the sequence to obtain total acid volume of 50.0 ml. Shake the mixture gently and allow the layers to separate. Draw off approx. 40 ml of the lower acid layer filtering through Whatman No. 1 filter paper. Pipet 10.0 ml of the acid extract into a 30 ml screw-cap tube to be ready for the Bratton-Marshall reaction (10 gm tissue equivalent). Pipet 25.0 ml of the acid extract into a 250 ml separatory funnel (25 gm tissue equivalent). Add 30 ml of saturated sodium citrate solution and adjust the pH to 6.5-6.7 with 5N NaOH. Extract the solution with 50 ml methylene chloride and allow the layers to separate. Collect the lower layer in a

250 ml round bottom flask. Repeat the extraction with an additional 20 ml methylene chloride. Evaporate the combined extracts to dryness on a rotary evaporator at 50°C. Dissolve the residue in 2.0 ml mobile phase and inject 50 µl into the liquid chromatograph.

Direct Standard Curve

To establish recoveries, prepare a direct standard curve for sulfamethazine. Evaporate to dryness in a 100 ml round bottom flask 0.25, 0.5, 1.0 and 2.5 ml of sulfamethazine standard solution (5 µg/ml) corresponding to 50, 100, 200 and 500 ppb levels. Dissolve the residue in 2.0 ml mobile phase and inject 50 µl into the liquid chromatograph.

Liquid Chromatography

Prepare the mobile phase consisting of 25% methanol in 0.01 M ammonium acetate and adjust the pH to either 6.7 or 3.5. Operate the system at ambient temperature with a mobile phase flow of 2.0 ml/min. and the detector set at 254 nm. Fix the chart speed on the reporting integrator at 0.5 cm/min., attenuation at 128 and slope sensitivity at 0.5.

Bratton-Marshall Reaction

Prepare all reagents fresh daily. Add 1.0 ml of 0.1% sodium nitrite to a 10.0 ml portion of sample acid extract or standard solution (10.0 ml of 100 ppb sulfamethazine in IN HCl). Mix well and allow to stand for 3 minutes. Add 1.0 ml of 0.2% ammonium sulfamate, mix well and allow to stand 2 minutes. Add 1.0 ml of 0.1% NED, mix well and allow to stand for 30 minutes for color development. Compare the sample with 100 ppb sulfamethazine standard. Presence of a pink color indicates a positive sample.

Thin Layer Chromatography

Extract Bratton-Marshall reaction mixture with 6 ml 1-butanol in 30 ml screw-cap tube. Allow the layers to separate and remove the top layer with a Pasteur pipet and transfer into a 50 ml round bottom flask. Repeat the extraction with 3 ml 1-butanol and evaporate the combined extracts on a rotary evaporator at 50°C. Dissolve the residue in 50 µl methanol and spot 10 µl on a TLC plate. Include on the same plate both the samples and sulfamethazine standard. Develop the plate in a mobile phase composed of 72 ml acetone, 21 ml hexane, 9 ml methanol, 10 ml 1-butanol and 10 ml ammonium hydroxide for approximately 2.5 hours (15 cm), remove from the sandwich apparatus, air dry and examine. The presence of pink spots with the same Rf value as the standard confirms the presence of sulfamethazine residue in the sample.

RESULTS AND DISCUSSION

Because of the possible presence of interfering endogenous substances, the Bratton-Marshall reaction can only be useful for the qualitative detection of sulfonamide residues in tissues. Moreover, some sulfonamide esters like succinylsulfathiazole and phthalylsulfacetamide, will not produce color with the Bratton-Marshall reaction unless they are hydrolyzed first to parent sulfonamides. Attempts to employ this reaction for quantitative measurements are therefore inherently imprecise. The Bratton-Marshall reaction is used in this laboratory only to indicate the presence of a sulfonamide residue; quantitation measurements are done by means of HPLC and confirmation by TLC.

The extraction procedure is essentially similar to that of Goodspeed et al. (7), but no derivatization steps are required for HPLC and therefore substantial saving of time is achieved. Initial extraction with ethanol resulted in higher recovery and less interference than with the acetone extraction. The 1N HCl

extraction step makes this method useful for both the free and conjugated sulfonamides.

HPLC is capable of separating all 14 sulfonamides approved for use in swine in Canada. The retention volumes relative to sulfamethazine are given for two pH levels (6.7 and 3.5) in Table 1. Sulfamethazine is eluted in both at approximately 14 ml mobile phase which corresponds to 7 minutes retention time with 2.0 ml/min flow rate. For practical purposes, sulfonamides with retention times less than 3 minutes (approx. 0.4 RRV) in either system cannot be measured by this method because of the background interference of polar tissue compounds. This affects two of the sulfonamides: sulfaguanidine and sulfanilamide. So far, the method has been verified only for sulfamethazine residues in pork kidney, liver and muscle. More work is needed for other sulfonamides. HPLC detects both sulfonamide esters, with pH 3.5 being the more practical one. Because the great majority of positive samples found contain sulfamethazine, the method was adapted mainly for this drug.

Data for the standard curves and recoveries for each tissue at 50, 100, 200 and 500 ppb levels are listed in Table 2. The standards were analyzed on two identical HPLC systems using two reverse phase columns with good reproducibility. Linear regression parameters were calculated and are given in Table 3. The values for the slopes ($\pm$ 95% confidence interval) were obtained by forcing the curve through the origin, since the regression with y intercept has shown that this intercept was not significantly different from 0. The HPLC system was linear for up to 5 μg of sulfamethazine standard on column.

The relative mobility (Rm's) values for derivatized sulfonamide standards are listed in Table 1. The Rf for derivatized sulfamethazine is 0.58. This TLC procedure is used to confirm the identity of the sulfa drug residue after detection by HPLC.

The method was verified by a 150 sample survey of pork tissues (50 each kidney, liver, muscle) purchased at the local supermarkets. About 4% contamina-

TABLE 1

Relative retention volumes (RRV's) of 14 sulfonamide standards on RP-10A reverse phase column at pH 6.7 and 3.5 and relative mobilities (RM's) of derivitized sulfonamides on silica gel plates.

Sulfonamide	Relative Retention Volume pH 6.7	pH 3.5	Relative Mobility
Sulfamethazine	1.00[a]	1.00[b]	1.00[c]
Sulfathiazole	0.52	0.54	1.19
Sulfamerazine	0.63	0.71	0.98
Sulfaquinoxaline	1.89	7.08	1.29
Sulfapyridine	0.60	0.60	1.16
Sulfadiazine	0.38	0.50	0.88
Sulfanilamide	0.27	0.27	1.53
Sulfadimethoxine	2.07	5.02	1.26
Sulfaethoxypyridazine	2.47	2.63	1.29
Succinylsulfathiazole	0.32	0.89	-
Phthalylsulfacetamide	0.22	1.36	-
Sulfachloropyridazine	0.48	1.27	1.22
Sulfadoxine	0.73	1.80	1.06
Sulfaguanidine	0.25	0.26	1.29

a absolute retention volume for sulfamethazine at pH 6.7 is 14.24 ml

b absolute retention volume for sulfamethazine at pH 3.5 is 14.04 ml

c Rf for derivatized sulfamethazine is 0.58

TABLE 2

Standard curve data and recoveries for sulfamethazine in pork kidney, liver and muscle in two HPLC systems.

Added ppb	Peak Area counts x 1,000		Std. dev.		Rel. Std. dev., %		Rec'd, %	
			Kidney					
50	12.6[a]	13.0[b]	1.6[a]	0.6[b]	12.7[a]	4.6[b]	90.3[a]	90.8[b]
100	23.3	23.1	1.8	1.5	7.7	6.5	76.5	73.5
200	49.8	50.9	3.1	2.6	6.2	5.1	79.5	78.2
500	118.2	119.8	9.5	9.4	8.0	7.8	73.9	71.8
			Liver					
50	10.6	10.9	1.4	1.6	13.2	14.7	78.9	80.1
100	22.0	23.0	2.0	2.3	9.1	9.9	74.4	75.5
200	44.2	47.4	4.1	4.3	9.3	9.1	71.5	73.8
500	120.0	126.4	7.8	8.5	6.5	6.7	75.3	76.2
			Muscle					
50	11.7	12.8	1.7	1.9	14.2	15.1	83.6	88.9
100	24.4	25.7	1.9	2.3	7.6	8.8	80.2	81.8
200	47.6	49.8	3.3	1.7	7.0	3.5	76.0	76.8
500	120.7	125.5	8.0	8.4	6.6	6.7	75.4	75.2

[a] HPLC system 1 data

[b] HPLC system 2 data

TABLE 3

Linear regression parameters for sulfamethazine in pork kidney, liver and muscle in two HPLC systems.

Tissue	Slope[a]		Regression Coefficient[b]	
Kidney	238.4 ± 7.7[c]	241.4 ± 7.5[d]	0.995[c]	0.995[d]
Liver	236.6 ± 7.3	249.7 ± 7.7	0.995	0.995
Muscle	240.9 ± 6.5	250.9 ± 6.5	0.996	0.996

[a] slope ± 95% confidence interval

[b] r^2 value

[c] HPLC system 1

[d] HPLC system 2

tion by sulfamethazine at levels up to 3 ppm was found. None of the other sulfa drugs listed in Table 1 have been detected in any of the tissues analyzed. For illustration, chromatograms of negative and positive samples in each tissue are shown in Figure 1.

The above described method has several advantages over those previously reported (6,7), namely, improved extraction procedure, recovery and separation, as well as the obviation of derivatization and incorporation of an additional confirmation test. In our laboratory, the entire procedure is completed in one day, as compared to two days required for most other methods.

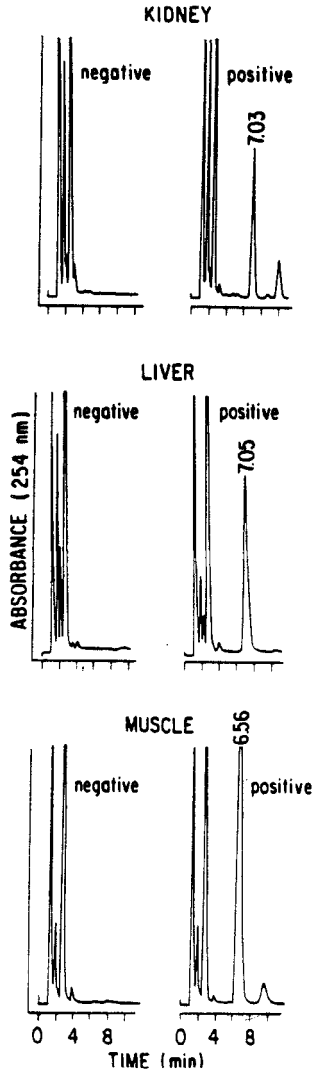

Figure 1. Chromatograms of negative and positive samples of pork kidney, liver and muscle. The positive values for kidney, liver and muscle are 830 ppb, 1.7 ppm and 3.0 ppm respectively.

ACKNOWLEDGEMENTS

The authors would like to thank the following companies for gifts of sulfa drug standards: American Cyanamid (sulfaethoxypyridazine), Bate Chemicals (succinylsulfathiazole), Burroughs Wellcome (sulfadoxine) and Squibb Canada (sulfachloropyridazine).

REFERENCES

1. Code of Federal Regulations, 1979, 21, part 556.
2. Bratton, A.C. and Marshall, E.K., J. Biol. Chem., 128, 537 (1939).
3. Tishler, F., Sutter, J.L., Bathish, J.N. and Hagman, H.E., J. Agric. Food Chem., 16, 50 (1968).
4. Phillips, W.F. and Trafton, J.E., J. Assoc. Off. Anal. Chem., 58, 44 (1975).
5. Woolley, Jr., J.L., Murch, O. and Sigel, C.W., J. Assoc. Off. Anal. Chem., 61, 545 (1978).
6. Johnson, K.L., Jeter, D.T. and Claiborne, R.C., J. Pharm. Sci., 64, 1657 (1975).
7. Goodspeed, D.P., Simpson, R.M., Ashworth, R.B., Shafer, J.W. and Cook, H.R., J. Assoc. Off. Anal. Chem., 61, 1050 (1978).