Journal of Chromatography, 555 (1991) 315–320
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 442

Short Communication

Development of a screening method for five sulfonamides in salmon muscle tissue using thin-layer chromatography

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(First received February 13th, 1991; revised manuscript received May 7th, 1991)

ABSTRACT

A thin-layer chromatographic (TLC) method was developed for the analysis of five sulfonamides [sulfadiazine (SDZ), sulfamerazine (SMRZ), sulfamethazine (SMTZ), sulfadimethoxine (SDMX) and sulfapyridine (SP)] in salmon muscle tissue. "Matrix solid-phase dispersion" was employed whereby the tissue sample was ground with C_{18}-derivatized silica gel. This material was packed into a column and washed with 10% toluene in hexane (discarded) followed by dichloromethane which was evaporated. The residue was chromatographed on a high-performance TLC plate using ethyl acetate–*n*-butanol–methanol–aqueous ammonia (35:45:15:2, v/v). Sulfonamides were detected after spraying the plate with a solution of fluorescamine. Method parameters were determined by analyzing spiked salmon muscle tissue samples. The method detection limits at the 99% confidence level were 0.11, 0.44, 0.07, 0.13 and 0.13 ppm for SDZ, SMRZ, SMTZ, SDMX and SP, respectively. The lowest-detectable levels were approximately 0.04 ppm for SDZ, SMTZ, SDMX and SP, and 0.10 ppm for SMRZ. The average recoveries of analytes were 61, 63, 60, 63 and 57% for SDZ, SMRZ, SMTZ, SDMX and SP, respectively, and were found to be analyst-dependent. The method was found to give linear detector responses for all analytes over spiking levels ranging from 0 to 2 ppm.

INTRODUCTION

The objective of this work was to develop a rapid screening method for the fives sulfonamides sulfadimethoxine (SDMX), sulfadiazine (SDZ), sulfamerazine (SMRZ), sulfamethazine (SMTZ) and sulfapyridine (SP) in salmon muscle tissue. Several methods were reported for the analysis of sulfonamides in other animal tissue using solvent extraction with ion exchange [1] and reveresed-phase column cleanup [2], and also using ion-pair extraction [3]. The major problem with these methods when applied to salmon muscle tissue was the appearance of a pigmented (orange) oil in the final extract, as well as inconsistent recoveries for the above group of sulfonamides.

A method reported by Long *et al.* [4], for the analysis of sulfonamides in pork

0021-9673/91/$03.50

using "matrix solid-phase dispersion"[5] appeared more promising. This paper reports the results of the application of this method to salmon muscle tissue.

EXPERIMENTAL

Materials

Sulfonamides were obtained from Sigma (St. Louis, MO, USA) and Burroughs Wellcome (Kirkland, Canada). Stock (1000 ppm) solutions were prepared as follows: SDZ: Dissolve 0.05 g in 15 ml methanol plus three drops concentrated ammonia. Dilute to 50.0 ml with methanol; SMRZ: dissolve 0.05 g in 15 ml methanol plus 1 ml deionized water; dilute to 50.0 ml with methanol; SMTZ, SDMX and SP: dissolve 0.05 g in 50.0 ml methanol. Working solutions were prepared by diluting the stock solutions with methanol. C_{18}-derivatized silica gel (40 μm, Analytichem, Harbor City, CA, USA) was washed twice with hexane, dichloromethane and methanol. In each wash, the material was placed in a beaker and enough solvent was added to make a free-flowing slurry. This was filtered, and the damp material was placed in the beaker for the next wash. The second dichloromethane slurry was allowed to sit for 20 min to overnight before filtering. After the last methanol wash, the material was partially airdried, so that is was clumpy, but not dripping with methanol, and not a free-flowing powder. This material was placed in a foil-covered beaker and used immediately. Plastic columns were supplied by Brinkman Instruments (Rexdale, Canada); 10-ml plastic syringe barrels may also be used. High-performance thin-liquid chromatographic (HPTLC) plates (Merck; silica gel 60 F_{-254}, 0.25 mm, 10 × 20 cm) were supplied by BDH, Vancouver, Canada. Fluram solution was prepared by dissolving 0.01 g of fluorescamine (Sigma, St. Louis, MO, USA) in 100 ml of acetone and was stored in a freezer. All solvents were HPLC grade. Farmed salmon muscle tissue was obtained from a local market. (We found that wild salmon tissue gave a higher background.)

Extraction procedure

Salmon muscle tissue (0.50 ± 0.08 g) was placed in a glass mortar (*ca.* 236 ml). Spiking solution (10–100 μl) was placed on the tissue (methanol on tissue blanks). One "scoop" (2 g dry weight; *ca.* 5 ml) of methanol-damp C_{18} material was added and the sample was ground with the pestle. After grinding for approximately 20 s the mortar contents were scraped into the center (to ensure thorough mixing) and grinding was continued for approximately 30 s. The contents of the mortar were transferred to a Brinkman column (a 10-ml plastic syringe barrel may also be used) and tightly compacted with a glass rod (4-mm diameter).

The column was placed on a vacuum manifold and washed with 8 ml of 10% toluene in hexane ($\approx$ 1 drop/s; discard), and aspirated dry. A glass test tube, containing a folded Wathmann No. 1 filter paper (4.25 cm) inserted $\approx$ 2 cm below the top of the tube, was placed under the column, and the analytes were eluted with 8 ml of dichloromethane ($\approx$ 1 drop/s). The filter paper served to catch any C_{18} material from the column. (In-line PTFE syringe filters clogged when dichloromethane was added.) The dichloromethane eluate was transferred to a plastic centrifuge tube (15 ml) and evaporated to dryness under a stream of nitrogen using a 40°C water bath. The wall of the tube was washed wit 2 × 1 ml methanol; each wash was evaporated to dryness.

TLC analysis

Methanol (50 μl) was added to the sample residue and the contents were vortex-mixed (10 s) and sonicated (5 min). The solution (*ca.* 7 μl) was spotted on an activated (65°C, 12 h) HPTLC plate using a glass capillary tube (75 mm × 0.56 mm I.D.). During spotting the plate was warmed to *ca.* 70°C. The sports (diameter less than 4 mm) were "focused" by developing the plate in a methanol bath to ≈ 2 mm above the origin. The plate was airdried in a fume hood for ≈ 10 min. During this time the chromatography bath was prepared: ethyl acetate–*n*-butanol–methanol–aqueous ammonia (30%) (35:45:15:2, v/v). The plate was developed to a height of 8.5 cm, airdried for *ca.* 10 min (fume hood), and sprayed evenly (side-to-side motion) with Fluram solution, such that the plate was saturated, but not dripping. The sprayed plate was airdried for *ca.* 30 min and scanned with a Camag II densitometer (equipped with an IBM XI-compatible computer) in the fluorescence mode (366 nm excitation). The dried plate could be covered with a glass plate and stored in a freezer overnight before scanning.

Quantitation

The method detection limit (MDL) was determined from the equation

$$\text{MDL} = St$$

where t is the students' "t" value appropriate for the desired confidence level and number of observations ($t = 3.143$ for $n = 7$, and 99% confidence level [6]), and S is the standard deviation of the analytical results of a group of samples spiked at one level. Seven tissue samples were spiked with all analytes at 0.1 ppm, and analyzed concurrently with seven samples spiked with methanol only (tissue blanks). Analyte peak arreas were corrected for tissue interferences by subtracting the average ($n = 7$) peak areas of the interferences found in the tissue blanks.

Recoveries of analytes from salmon muscle tissue were determined using a single-point calibration method. A standard containing all five sulfonamides was spotted on the same plate as the samples, such that groups of three to four samples were bracketed by pairs of standards spots. The amounts of analytes in each standard spot corresponded to that of 100% recovery from the spiked tissue samples.

RESULTS AND DISCUSSION

Chromatography

Baseline resolution of all analytes was achieved at a tissue spiking level of 0.1 ppm as shown in Fig. 1. The resolution of SDMX and SP was sensitive to the age of the TLC bath. Best results were obtained if the bath was prepared just before use. A tissue interference was observed for SMRZ (Fig. 1) which resulted in a higher detection limit for this analyte. Smaller interferences were observed for SDMX and SP.

MDL

With the exception of SMRZ, MDL values ranged from 0.07 to 0.13 ppm (Table I). The high MDL of SMRZ (0.44 ppm) was due to the tissue interference observed for this analyte (Fig. 1), the area of which was found to be quite variable in

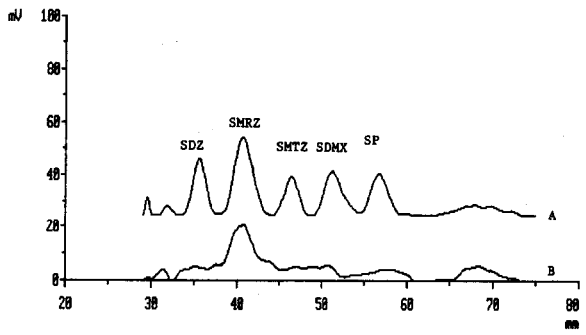

Fig. 1. Chromatograms (densitometer traces) of spiked salmon muscle tissue samples. (A) Spiking level = 0.1 ppm (all analytes); (B) 0 ppm (tissue blank). Detection at 366 nm.

the tissue blanks (coefficient of varation = 79%). These MDL values were calculated using a high confidence level (99%). The lowest-detectable levels, on the other hand, were approximately 0.04 ppm for SDZ, SMTZ, SDMX and SP, and 0.1 ppm for SMRZ.

Before scanning, plates were visually assessed under long-wavelength UV light. Spiking levels down to approximately 0.1 ppm could be observed in this manner.

Recovery

Recoveries of analytes from salmon muscle tissue were determined at two spiking levels: 0.5 and 2.0 ppm. with five replicates at each level. Recoveries did not vary significantly with spiking level. The overall average recoveries ranged from 57 to 63% for the five sulfonamides (Table I). On closer examination, it was found that recovery was analyst-dependent. The steps of sample grinding and column compacting were performed by two analysts, denoited as A and B. Analyst A used a larger and heavier pestle, and was also physically larger and heavier than analyst B. Recoveries of ana-

TABLE I

ANALYTICAL RESULTS FOR THE FIVE SULFONAMIDES IN SPIKED SALMON MUSCLE TISSUE

Compound	MDL[a] (ppm)	Correlation coefficient[b]	Average recoveries[c]		
			Analyst A ($n=5$)	Analyst B ($n=5$)	Overall ($n=10$)
SDZ	0.11	0.983	73±7	50±3	61±12
SMRZ	0.44	0.983	76±7	50±3	63±14
SMTZ	0.07	0.949	71±5	49±2	60±12
SDMX	0.13	0.858	72±8	54±3	63±11
SP	0.13	0.946	69±6	45±3	57±12

[a] Calculated at the 99% confidence level.
[b] Spiking levels: 0.10, 0.20, 0.30, 0.40, 1.0, 2.0 ppm. Two replicate samples analyzed per level.
[c] See text.

lyst A were consistently higher than those of analyst B (Table I). The average recoveries of analyst A were approximately 10% higher than the overall averages, ranging from 69 to 76% for the five sulfonamides (Table I). It was also observed that the extracts of analyst A, after evaporation of dichloromethane, contained slightly less fat/oil than those of analyst B.

It was speculated that analyst A applied a larger force to the pestle during grinding, resulting in more efficient distribution of the tissue sample over the C_{18} surface, and more efficient partitioning of the analytes (and endogenous substances) between the solvent and stationary phase furing column chromatography. Recoveries were not improved by using a more polar elution solvent (20% ethyl acetate in dichloromethane).

In the analysis of pork tissue (results not shown) it was found that recovery was influenced by the methanol content of the C_{18} material, and by the type of mortar and pestle used. Lower recoveries were observed using: (1) dry (free-flowing) C_{18} compared to methanol-damp material, and (2) a procelain mortar and pestle compared to a glass apparatus. In addition, it was shown that the duration of grinding (between 30 s and 2 min) did not affect the recovery of sulfonamides from prork tissue. These results were not validated for salmon musle tissue.

Recoveries of the five sulfonamides from wild trout (a salmonid) musle tissue were found to be 10–20% higher than from salmon muscle tissue (data not shown). This may be related to the relatively high cholesterol level in salmon muscle tissue [7], although its higher fat/oil content [8] may also be important.

Precision

The observed analyst dependency of recovery affected the precision of the results. The standard deviations of average recoveries of samples processed by a single analyst ranged from 2 to 8% (average = 5%), whereas the overall standard deviations of both sets of recoveries (analysts A and B) were significantly higher, ranging from 11 to 14% (average = 12%) (Table I).

Method linear range

Detector response was found to be linear for eacht analyte over a spiking range of 0.10 to 2.0 ppm, with correlation coefficients ranging from 0.858 to 0.983 (average = 0.944) (Table I).

ACKNOWLEDGEMENTS

This work was funded by the Department of Health and Welfare. Discussions with Dr. S. A. Barker were very helpful during method development work. Janet Milton assisted with the preparation of this manuscript.

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