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Note

Rapid determination of five sulphonamides in swine tissue by high-performance liquid chromatography

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Sulphonamides are used in veterinary practice as antibacterial agents. In 1973 a tolerance level of 0.1 mg kg^{-1} for residues of these drugs in animal tissues was set in the U.S.A. As microbiological methods are not sensitive enough to detect sulphonamides at such a low level, many methods have been developed for chemical analysis of sulphonamides in food of animal origin[1–8]. However, most of these involve time-consuming steps such as liquid–liquid partition and evaporation of large volumes. For this reason a rapid screening method has recently been developed for the determination of five sulphonamides in concentrations as low as 0.05 mg kg^{-1} in animal tissue[9,10]. This method includes a combined clean-up and concentration procedure by means of a solid-phase extraction column, *i.e.*, a Sep-Pak® silica cartrdige. The purified extracts are then subjected to thin-layer chromatography (TLC).

A rapid clean-up procedure has also been developed[11] for the determination of sulphamethazine by high-performance liquid chromatography (HPLC). In this method a dichloromethane extract of the tissue is purified and concentrated on a combination of a silica and a C_{18} Sep-Pak® cartridge. However, this method was found to be unsuitable when applied to the simultaneous determination of five sulphonamides, *i.e.*, sulphanilamide (SA), sulphamethazine (SMZ), sulphaquinoxaline (SQ), sulphadoxine (SDX) and sulphadiazine (SD), due to their different solubilities and pK_a values.

This paper describes an HPLC method for a rapid quantitation of the above five sulphonamides in swine tissue. In this procedure, both the clean-up and concentration steps are realized by the use of a cation-exchange solid-phase extraction (SPE) column. As this clean-up is based on the amphoteric properties of these compounds, it is expected that the extension of this procedure to other sulphonamides will cause no difficulties.

MATERIALS AND METHODS

Reagents and chemicals

All solvents used were reagent grade unless mentioned otherwise. Water was purified via Milli-Q® (Millipore, Bedford, MA, U.S.A.). Acetic acid (99%) and ammonium acetate (HPLC grade) were from Baker (Phillipsburg, NJ, U.S.A.), methanol

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and acetonitrile (both HPLC grade) from Rathburn (Walkerburn, U.K.) and chloroform and acetone (both Chrom AR) from Mallinckrodt (St. Louis, MO, U.S.A.).

Filter-paper circles (S & S 589.1, diameter 90 mm), were from Schleicher and Schüll (Dassel, F.R.G.) and the ammonia cylinder (99.9%, 170 g) was from Matheson (Oevel, Belgium). SA, SD, SMZ and SQ sodium salt were from Sigma (St. Louis, MO, U.S.A.) and SDX was from Hoffman-La Roche (Basle, Switzerland).

Sulphonamide standard solution was prepared by dissolving 100 mg of each sulphonamide in 100 ml of methanol. Working standards for HPLC were prepared in the range of 0–20 μg per ml of eluent by diluting the standard solution in the mobile phase solvent. Spiking solutions containing 0.05, 0.1 and 0.5 mg ml^{-1} methanol were prepared by diluting the standard solution in methanol.

Aromatic sulphonic acid cation-exchange columns (3 ml) were obtained from Baker. Just before use the column was pretreated by passing 2 × 3 ml of hexane and followed after drying by 2 × 3 ml of chloroform–acetone (1:1, v/v) containing 5% acetic acid. After this final treatment the column should not allowed to run dry.

The mobile phase solvent was acetonitrile–10 mmol l^{-1} ammonium acetate buffer pH 4.6 (3:7, v/v).

Samples

Swine kidney and ham muscle were used. Visible fat and collagen were removed as far as possible. If the tissue pH exceeded 5.5, it was lowered to this value by use of an acetic acid–water (1:10, v/v) solution. Ground tissue samples were spiked at levels of 0.05, 0.1 and 0.5 mg kg^{-1} at least 15 min before extraction by the procedure described below.

Apparatus and chromatographic conditions

The instruments used were a Moulinette homogenizer (Moulinex, Gouda, The Netherlands), a Bransonic® B-221 ultrasonic bath (Branson Europe, Soest, The Netherlands) equipped with a tray insert and filled with water, a Vortex mixer (Scientific Industries, Bohemia, U.S.A.) and a table centrifuge (Type TJ6; Beckmann Instruments, Geneva, Switzerland). In order to operate several cartridges simultaneously, a vacuum manifold (Baker) was used. It was connected, via a filtration flask, to a water aspirator. The extraction column was connected to a 75-ml reservoir equipped with an adaptor (Baker). For sample elution a collection rack was inserted in the vacuum manifold basin. For HPLC, a LKB 2150 pump (Bromma, Sweden) equipped with a 20-μl Rheodyne 7125 sampling valve (LKB) and a 2138 Uvicord S detector operated at 254 nm and equipped with an 8-μl HPLC cell (LKB) was used. A stainless-steel column (250 × 4 mm) containing 8-μm CP® SpherC_8 (Chrompack) was used in connection with a guard column (75 × 2.1 mm) packed with RP (Chrompack). The chromatograph was operated at ambient temperature. Peak heights were measured with a SP4270 printer/plotter integrator (Spectra-Physics, San Jose, CA, U.S.A.).

Sample preparation

Extraction. Approximately 10 g of ground tissue (pH *ca.* 5.5) were weighed in a 100-ml beaker. A 25-ml volume of chloroform–acetone (1:1) was added. After stirring thoroughly with a glass rod, the beaker was covered and placed on the tray

insert in the ultrasonic bath for 10 min. The temperature of the water in the bath was kept below 40°C. The solvent was poured off through filter-paper. The extraction was repeated twice and the solids were rinsed with an additional 25 ml of chloroform–acetone (1:1). A 5-ml volume of acetic acid was added to the combined extracts (volume 70–95 ml).

Clean-up. The total extract was passed through the pretreated ion-exchange extraction column (connected with the 75-ml reservoir) at a rate of *ca.* 8–10 ml/min by suction through the vacuum manifold. The column was washed successively with 5 ml of water and 5 ml of methanol. It was then removed from the manifold and the 75-ml reservoir and was dried in a stream of air for 10 min. A stream of ammonia vapour was passed through the column for 10 min.

The column was placed on the vacuum manifold, and in the rack positioned in the vacuum manifold basin a 3.5-ml polypropylene collection tube was placed under the extraction column. A 3-ml volume of methanol was added to the column, without the application of a vacuum ('moistening'), after which the sulphonamides were eluted from the column by suction of the methanol through the vacuum manifold.

The eluate was evaporated to dryness in a stream of air. The residue was dissolved in 0.5 ml of the mobile phase solvent using a Vortex mixer for 15 sec. The solution was centrifuged for 10 min at 2000 *g* and the supernatant was used for chromatography.

Chromatography. 20-µl Aliquots of the sample and standard solutions were injected by means of the loop injector. Samples were eluted isocratically at a flow-rate of 1.5 ml/min.

RESULTS AND DISCUSSION

Spiking studies

Recovery experiments were carried out on muscle tissues at 0.05, 0.1 and 0.5 mg kg^{-1} spiking levels. Six samples of the ground tissue were spiked at each level. The samples were subjected to HPLC analysis in duplicate. Six replicate blank samples were analysed. The results are presented in Table I.

In these recovery experiments all five sulphonamides were present together. This situation will obviously not occur in practice, but the results do show the high recovery of the compounds as well as the easy separation from each other and from other muscle compounds under the described HPLC conditions. All peaks of these endogenous muscle compounds appear on the chromatogram during the first 2 min (see Fig. 1). This enables both quantitation and identification in a sample containing an unknown sulphonamide. It should be noted that, if the presence of a sulphonamide is demonstrated in an extract from a treated animal, the parent sulphonamide peak may in principle be accompanied by a metabolite peak. For example, in the case of tissue from SMZ-treated swine, a peak of the mean metabolite, N^4-acetylsulphamethazine, is found at a retention time, t_R, or 3.2 min together with the parent peak, t_R = 4.3 min, in muscle and kidney tissue. The recovery of this metabolite from a standard solution in acidified chloroform–acetone using the described clean-up procedure is higher than 80%. Therefore, since it is known[12] that this metabolite is extracted nearly quantitatively from swine tissue by means of chloroform–acetone

TABLE I

RECOVERY OF SULPHONAMIDES FROM SPIKED SWINE MUSCLE

Sulphonamide	*Added (mg kg^{-1})*	*Found (mg kg^{-1})*	*Standard deviation (n = 6)*	*Coefficient of variation (%)*	*Recovery (%)*
SA	0.05	0.044	0.002	3.6	88
SD	0.05	0.041	0.002	5.6	82
SMZ	0.05	0.046	0.001	2.8	92
SDX	0.05	0.044	0.002	4.7	87
SQ	0.05	0.048	0.002	4.7	96
SA	0.1	0.082	0.003	3.8	82
SD	0.1	0.083	0.003	3.7	89
SMZ	0.1	0.091	0.004	4.1	91
SDX	0.1	0.087	0.005	5.4	87
SQ	0.1	0.084	0.002	3.5	84
SA	0.5	0.470	0.015	3.1	94
SD	0.5	0.430	0.003	0.8	86
SMZ	0.5	0.440	0.006	1.4	88
SDX	0.5	0.450	0.018	3.9	90
SQ	0.5	0.420	0.013	3.0	84

(1:1, v/v), it is expected that this procedure will also prove suitable for a simultaneous determination of SMZ and its N^4-acetyl metabolite in animal tissue. Such a procedure will be investigated.

In early experiments, variable recoveries of SDX and SQ were sometimes

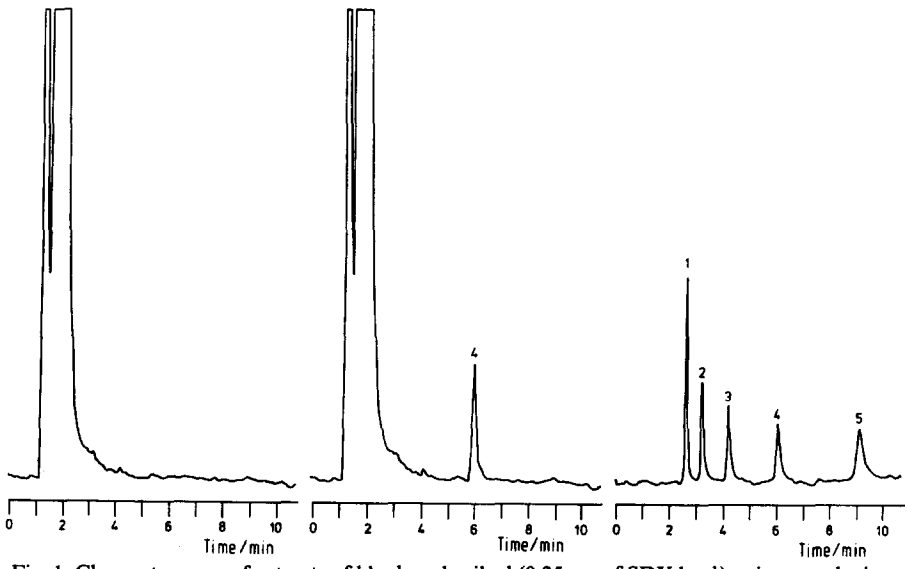

Fig. 1. Chromatograms of extracts of blank and spiked (0.25 mg of SDX kg^{-1}) swine muscle tissue and of a standard solution of sulphonamides. For conditions see text. Absorbance range setting: 0.05 a.u.f.s. Peaks: 1 = SA; 2 = SD; 3 = SMZ; 4 = SDX; 5 = SQ.

found, particularly from muscle samples of somewhat higher pH, *e.g.*, meat of the Dark Firm Dry type). To overcome this problem, the pH of the muscle was measured before extraction and lowered, if necessary, to *ca.* 5.5 by the addition of diluted acetic acid. Nevertheless, even with this precaution, occasionally low recoveries are found from kidney tissue. This phenomenon cannot yet be explained. The described procedure can be used for kidney tissue, providing recovery experiments are performed in duplicate for each sample. Most recovery values for kidney tissues are also in the range of 80–90% for a spiking range of 0.05–0.5 mg kg^{-1}. The chromatogram for a blank kidney tissue shows more endogenous compounds in the first few minutes than are found in chromatograms of blank muscle tissues. Some small peaks with higher retention times are also observed, but they do not interfere with the determination of any sulphonamide under our conditions.

For HPLC the Petz method[2] was used. Normally, chromatography on a C_8 column and an acetonitrile–ammonium acetate ratio of 3:7 is most suitable (t_R 2.6 min for SA, 3.3 min for SD, 4.3 min for SMZ, 6.1 min for SDX and 9.2 min for SQ). However, when this eluent is used, SA is occasionally eluted on the slope of the last appearing peak of the endogenous compounds. It is therefore recommended that if in TLC screening[9] SA was found to be present, the eluent ratio be changed to 1:3 for SA quantification by means of HPLC. However, this change also causes a slower elution of the other sulphonamides, especially in the case of SQ (t_R 2.8 min for SA, 3.8 min for SD, 5.4 min for SMZ, 8.7 min for SDX and 15.1 min for SQ).

Extraction and clean-up

The extractant chloroform–acetone (1:1), used in several other methods[1,4,6] for sulphonamide determination, was found to be suitable in our sonication-aided extraction as well. This mode of extraction allows the simultaneous extraction of several samples, thereby also excluding cross-contamination[11].

The clean-up procedure based on silica and reversed-phase cartridges, as described for SMZ[11], was found to be impossible for other sulphonamides due to their different solubilities and pK_a values. Therefore a cation-exchange SPE column was used which enables the clean-up of all sulphonamides under consideration. From an acidified extract, all sulphonamides are completely retained on the SPE column. Elution may be performed with an alkaline buffer, however, in this case the eluate provided to be unsuitable for HPLC analysis. This problem was overcome by passing ammonia vapour through the column, and subsequently eluting with methanol. The eluate thus obtained contains a smaller amount of salts and can be evaporated to dryness more readily than an aqueous eluate. A possible explanation for the elution of the sulphonamides by an organic solvent could be that the ammonia deprotonates the sulphonamides, thus breaking the binding with the sulphonic acid column and permitting the elution with a small volume of solvent (in which the sulphonamides dissolve reasonably).

Before the ammonia vapour treatment, an intermediate wash with water and methanol was introduced to remove some retained compounds of the matrix. For muscle tissue this washing procedure was not strictly necessary, but for kidney extracts it proved to be essential. For all samples, however, the washing strongly diminishes the time required for evaporation of the methanol eluate.

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REFERENCES

1 A. J. Manuel and W. A. Steller, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 794.
2 M. Petz, *Z. Lebensm.-Unters.-Forsch.*, 176 (1983) 289.
3 M. H. Thomas, R. L. Epstein, R. B. Ashworth and H. Marks, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 884.
4 F. B. Suhre, R. M. Simpson and J. W. Shafer, *J. Agric. Food Chem.*, 29 (1981) 727.
5 A. J. Malenovski, C. J. Barnes and T. Fazio, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1386.
6 R. M. Simpson, F. B. Suhre and J. W. Shafer, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 23.
7 D. Jonas, G. Knupp and H. Pollmann, *Arch. Lebensmittelhyg.*, 34 (1983) 138.
8 B. Schlatterer, *Z. Lebensm.-Unters.-Forsch.*, 176 (1983) 20.
9 N. Haagsma, B. Dieleman and B. G. M. Gortemaker, *Vet. Q.*, 6 (1984) 8.
10 N. Haagsma, *Z. Lebensm.-Unters.-Forsch.*, (1985) in press.
11 N. Haagsma, R. J. Nooteboom, B. G. M. Gortemaker and M. J. Maas, *Z. Lebensm.-Unters.-Forsch.*, (1985) in press.
12 J. E. Matusik, Ch. J. Barnes, D. R. Newkirk and T. Fazio, *J. Assoc. Anal. Chem.*, 65 (1982) 828.