Journal of Chromatography, 503 (1990) 260–265
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM 22 130

Note

Capillary gas chromatographic determination of sulphadimidine in pork tissues

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(First received July 7th, 1989; revised manuscript received October 27th, 1989)

Sulphonamides are important drugs in veterinary practice for the prevention and treatment of diseases and for promotion of animal growth. Therefore, it is essential to monitor human foodstuffs for drug residues resulting from both the promotion and treatment. Many chromatographic methods, such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC) have been utilized for the determination of residual sulphonamides in animal tissues and comparisons of these methods have been published[1–3]. Owing to the large number of samples that must be tested for sulphonamides it is of the utmost importance that the method utilized should be rapid and practical with a sensitivity below 100 ppb. HPLC methods are now preferred[4], because in contrast to GLC[5,6] no derivatization steps are required and it is less complicated than TLC separations[7].

For the GLC procedure it is necessary to convert sulphonamides into suitable derivatives with good chromatography behaviour that are highly electronegative and amenable to sensitive electron-capture detection (ECD). The most common derivatization involves either a single-stage N^1-methylation of the polar acidic sulpha group with diazomethane[8–10] or N^1-methylation followed by acylation of the N^4-primary amino function with pentafluoroalkane carboxylic anhydride[11,12]. Our method uses sorption of the residual sulphadimidine N^1-(4,6-dimethyl-2-pyrimidyl) sulphanilamide from an organic extract on activated alumina[13] and N^1-extractive methylation[14], followed by N^4-acetylation with trifluoroacetic anhydride. This method should prove convenient for the determination of residual levels of sulphadimidine (SDM) with rapid derivatization steps by means of high-resolution capillary gas chromatography with ECD and the possibility of the gas chromatographic–mass spectometric (GC–MS) confirmation of sulphonamide residues.

EXPERIMENTAL

Chemicals and reagents

The solvents chloroform, dichloromethane, toluene (analytical-reagent grade) (Chemapol, Prague, Czechoslovakia) and acetonitrile (VEB-Laborchemie, GDR) were doubly distilled in an all-glass apparatus with added P_2O_5 and cleaned on a

0021-9673/90/$03.50

chromatographic column filled with activated silica (70–230 mesh) (Merck, Darmstadt, F.R.G.) and basic alumina (Lachema, Brno, Czechoslovakia). *n*-Heptane (spectroscopic grade) from Chemapol was dried for 48 h with $LiAlH_4$, distilled and cleaned on a chromatographic column filled with activated silica (Merck). Carbonate buffers, 0.2 *M* (pH 11) and 0.5 *M* (pH 9.5), were prepared from 0.2 *M* and 0.5 *M* solutions of $NaCO_3$ and $NaHCO_3$. Acetate buffer 1 *M* (pH 5.5), was prepared from 1 *M* acetic acid and 1 *M* sodium acetate solutions. Tetrabutylammonium hydroxide (TBAH) buffer (0.1 *M*) was prepared from tetrabutylammonium bromide (Fluka, Buchs, Switzerland) by neutralization with 0.1 *M* NaOH, diluted to volume in a volumetric flask with 0.5 *M* carbonate buffer (pH 5,5) and cleaned by extraction with 50 ml of dichloromethane and 2 × 50 ml of *n*-heptane. Neutral activated alumina (Brockman activity 1) was prepared from chromatographic-alumina (100–200 mesh) (Reanal, Budapest, Hungary) by activation for 6 h at 450°C. The reagents methyl iodide (gold label; Aldrich, F.R.G.) as a 0.16 *M* solution in dichloromethane,4-dimethylaminopyridine (4-DMAP) (Sigma, F.R.G.) a a 2 mg/ml solution in toluene–acetonitrile (95:5), trifluoroacetanhydride (TFAA) and octamethylcyclotetrasiloxane (D_4) were obtained from Fluka, hexamethyldisilazane (HMDS) from Lachema and sulphadimidine base from Spofa (Czechoslovakia).

Sample preparation and purification

To 5 g of homogenized tissue sample in a 50-ml glass centrifuge tube, 5 ml of acetate buffer (pH 5.5) and 10 ml of ethyl acetate–chloroform (1:1) were added. The mixture was stirred on an Ultra-Turrax at low speed. After a second addition of 10 ml of the extraction mixture to the sample, centrifugation was carried out at 250 *g*. The organic layer was dried by passage through a 3-cm layer of anhydrous Na_2SO_4 in a 5-ml pipette tip and transferred to a 60 × 8 mm sorption microcolumn with layers of 0.2 g of sea sand at the bottom, 0.3 g of activated alumina and 0.2 g of sea sand at the top, in a vacuum manifold flow-rate (*ca.* 2 ml/min). After application of 2 × 1 ml of chloroform to each microcolumn, the vacuum was increased. When the moisture on the outside walls disappeared, the column was eluted with 500 μl of carbonate buffer (pH 11).

Derivatization

N^1-Extractive alkylation. To a 100 μl of buffered effluent in 1-ml amber-coloured Reacti-Vials, 100 μl of TBAH solution in 0.5 *M* carbonate buffer was added. After the addition of 200 μl of 0.16 *M* methyl iodide solution in dichloromethane, the Reacti-Vials were firmly capped and heated for 10 min at 70°C in a dry-heated block, then throughly shaken in shaker for 20 min. Phase separation was effected by centrifugation at 250 *g*. A 100-μl bottom organic layer was withdrawn in to 3 ml Reacti-Vials and evaporated at 40°C under gentle stream of nitrogen.

N^4-Trifluoroacetylation. A 1-ml volume of mixture of 4-DMAP in toluene–acetonitrile (95:5) and 50 μl of TFAA were added to each dry residue. The vials were heated for 20 min at 70°C. After the reaction was completed[15], the vials were cooled to room temperature, 1 ml of 3% $NaHCO_3$ was added and the vials were shaken. After centrifugation at 50 *g*, 100 μl of the organic layer were withdrawn into 900 μl of *n*-heptane and 3 μl of each sample were then injected by an autosampler (ALS) into the capillary column in the splitless mode.

Calculations

A calibration graph was obtained using 12.5, 25.0, 125.0 and 250.0 ng/ml of underivatized SDM solution in the extraction mixture to give 50, 100, 500 and 1000 ppb levels for raw tissue samples. After the sorption and derivatization steps with 20 ml of this mixture four calibration measurements were made at each level. The equation for the resulting line ($P \leq 0.05$) was $y = 1.9845 \cdot 10^{-5}x - 0.1011$ ($r = 0.9975$). The external standard method on the peak area of SDM in unknown samples was employed to calculate their concentrations from the calibration graph.

Instrumental

Analyses were performed on a Hewlett-Packard 5880A level IV gas chromatography with split–splitless injection, a Model 7671A autosampler and a ^{63}Ni electron-capture detector. An Ultra 1 capillary column (25 mm × 0.2 mm I.D., 0.33 μm film thickness (Hewlett-Packard, Avondale, PA, U.S.A.) was connected to a 1 m × 0.2 mm I.D. deactivated retention gap by a butt connector (all from Supelco, Bellefonte, PA, U.S.A.) to the injection port. Data collection and integration were performed for a set 0.02-min peak width, attenuation 2 × 10, treshold 9 and chart speed 0.5 cm/min.

Instrumental conditions

The injection port and detector temperature were set at 200 and 350°C, respectively. The oven temperature programme was 80°C initial temperature, 1-min hold; 30°C/min to 290°C; 20°C/min to 320°C, 1-min hold; and post-column 325°C, 1-in hold.

The carrier gas was Ultra-pure helium (Messer and Griesheim, Austria) at a flow-rate of 0.3 ml/min, cleaned via an oxygen filter and molecular sieve 5A, head pressure 200 kPa, splitting ratio 1:50, septum purge 1 ml/min and splitless delay 1 min. The make-up gas was argon–methane (95:5) (Messer and Griesheim) at a flow-rate of 50 ml/min, regulated through a base flow unit (BFU 101; Chrompack, Middelburg, The Netherlands), inlet pressure 70 kPa, cleaned over activated charcoal and molecular sieve 5A in series.

The capillary column was conditioned at 300°C for 24 h, 5 × 80°C for 1 h and 5 × 320°C, 1-h hold. The solvent effect was determined with five 3-μl *n*-heptane injections with the model 7671A autosampler. The retention time for derivatized SDM under these conditions was 8.43 ± 0.00 min for base-horizontal separation.

RESULTS AND DISCUSSION

The accuracy and precision of the method for the determination of SDM in standard samples at two sensitivity levels are presented in Table I. The results for the retention time repeatibility and chromatograms for real samples (Fig. 1) indicate that the method is selective and sensitive. The splitless injection mode is a suitable technique for the determination of residual SDM in pork tissues. The source of errors arises from the separation and derivatization procedures. For separation we used solvent extraction from buffered samples excluding the influence of other endogenous substances from tissue matrix[16,17] on the accuracy and precision of the method.

The extraction recovery from tissues was determined by quadruplicate addition of SDM solvent solution to each homogenized tissue sample. The absolute recovery

TABLE I

ACCURACY AND PRECISION OF DETERMINATION OF SULPHADIMIDINE IN STANDARD SAMPLES

Results calculated as the means of six determinations.

Sensitivity for peak level (pg)	*Retention time (min) (mean ± S.D.)*	*Relative standard deviation (%)*	
		Intra-	*Inter-*[a]
5	8.427 ± 0.0013	1.01	7.99
100	8.426 ± 0.0015	3.46	6.76

[a] Calculated from four determinations.

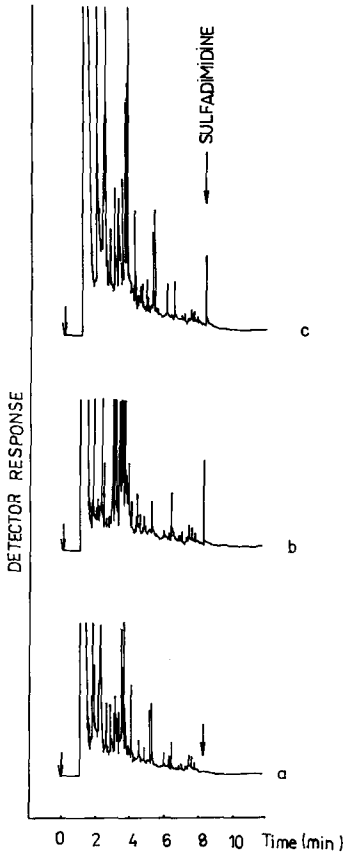

Fig. 1. Typical gas chromatograms of extracts carried through the procedure: (a) blank liver; (b) liver extract from 10th day after the end of 5 days treatment; (c) sulphadimidine standard, 0.05 $\mu g\ g^{-1}$.

TABLE II

RECOVERIES OF SULPHADIMIDINE FROM PORK TISSUES

Results calculated as the means of four determinations.

Sample	*SDM added to 1 g of tissue (ng)*	*SDM recovered after extraction (ng) (mean ± S.D.)*	*Recovery (%)*	*R.S.D. (%)*
Flesh (ham)	50	43.21 ± 4.04	87.08	9.27
Kidney	50	34.63 ± 1.68	69.26	4.86
Liver	50	40.21 ± 2.02	80.42	5.02

was calculated by comparing the calibration graph for SDM with results obtained using the described procedure with spiked samples (Tabe II). Our results at a spiking level of 50 ppb were lower than those with the HPLC method only for kidney[18]. For liver at a spiking level of 100 ppb and the some solvent extraction system a recovery 50.8% has been achieved[19] and for extraction with acetone–chloroform (1:1) a recovery of 84.3%[8]. The results of the recovery studies depend on the calibration procedure used and the polarity of the extraction system[20]. The large variations in the recovery studies show a significant matrix effect as a result of the higher fats and glucuronide content of organ samples. The lower recovery from pig kidney is probably due to the high adsorption of free SDM in this organ[21].

Our method, involving the sorption of residues on activated neutral alumina as a purification step, followed by rapid derivatization, is suitable for routine analysis. Under the operating conditions a detection limit of 10 ppb, corresponding to a minimum of 1 pg of derivatized SDM (at a signal-to-noise ratio > 10) was achieved. For this picogram detection limit its critical to have very pure solvents and a thoroughly deactivated injection liner. The method has been used for the determination of residual SDM in pork tissue after 5 days of continuous feeding with a dosage at the level of 50 mg of SDM per kilogram live weight (Table III). This method is rapid and enables

TABLE III

SULPHADIMIDINE RESIDUAL CONCENTRATION IN PORK TISSUES AFTER 5 DAYS OF CONTINUAL FEEDING WITH A DOSAGE AT THE LEVEL OF 50 mg OF SDM PER KILOGRAM LIVE WEIGHT

Results calculated as the means of two determinations, made 6, 10, 16 and 20 days after the end of 5 days treatment.

Sample	*SDM (ng/g) after day*			
	6	*10*	*16*	*20*
Liver	120	160	[a]	N.D.
Kidney	180	110	N.D.	N.D.
Lungs	100	80	50	N.D.
Flesh (ham)	60	N.D.	N.D.	N.D.
Blood	80	N.D.	N.D.	N.D.

[a] Below the detection limit of the method (10 ng/g).

ten samples to be prepared simultaneously. Over 100 injections of tissue extracts have been performed without changing the splitness liner.

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