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Comparison of the determination of four sulphonamides and their N^4-acetyl metabolites in swine muscle tissue using liquid chromatography with ultraviolet and mass spectral detection

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Abstract

A discharge-assisted LC–MS method has been developed and validated for the analysis of four sulphonamides (sulphathiazole, sulphadiazine, sulphamerazine and sulphadimidine) and their N^4-acetyl metabolites in the muscle of swine treated with Polysulpha-Complex, which contains all four drugs. The clean-up procedure developed involved chloroform–acetone extraction followed by Sep-Pak silica solid-phase extraction. In parallel a LC-UV method was validated using the same clean-up procedure. Blank tissue was fortified at levels between 20 and 100 μg/kg. [^{13}C]sulphadimidine was used as internal standard. The samples were analysed with thermospray LC–MS. The $[M+H]^+$ ion was the major ion in all cases and was employed for single-ion monitoring. The limits of detection (LOD) were below 25 μg/kg and the limits of quantification (LOQ) for most sulphonamides were ca. 100 μg/kg. Incurred muscle tissues were measured by both LC methods and the concentrations of the sulphonamides were found to be similar. However, the LC–MS procedure is more suitable for confirmatory analysis due to its specificity.

1. Introduction

The sulphonamides are a group of antibacterial agents commonly used in farm-animal feedstuffs for prophylactic and therapeutic purposes [1]. The structures of the eight compounds considered are shown in Fig. 1. EEC Regulation 675/92 [2] requires the provision of an analytical procedure for the determination in edible tissue which has a limit of quantification of 100 μg/kg [2]. It is essential that a degree of specificity is introduced to differentiate between the sulphonamides and their N^4-acetyl metabolites because of the large number of sulphonamides used in veterinary medicine.

A variety of methods have been published for the determination of sulphonamides in swine tissue and these include LC with UV detection [3–8], GC with electron-capture detection [5] and GC–MS after methylation [9,10]. The combination of LC with ion-spray MS has also

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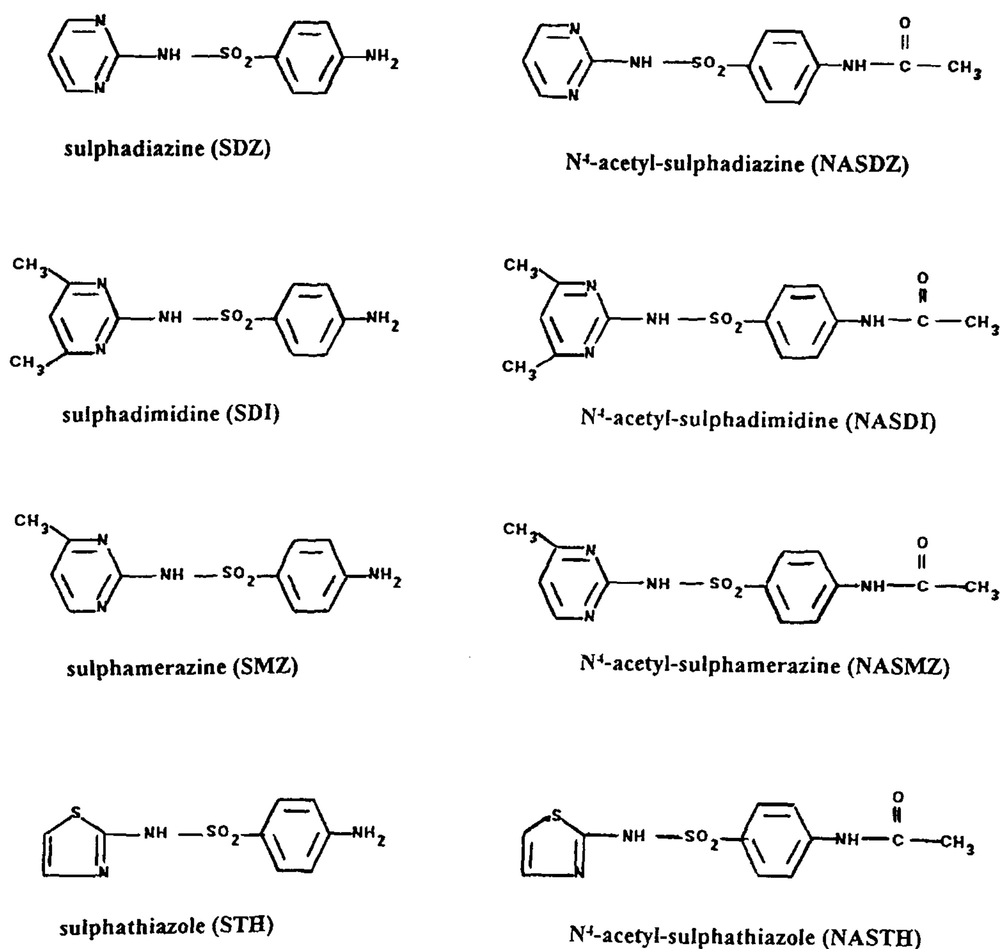

Fig. 1. Structure of the sulphonamides and their N^4-acetyl metabolites.

been considered by Pleasance et al. [11] for the determination of 21 sulphonamides in salmon tissue. Among the other LC–MS techniques considered was LC–thermospray (TSP)-MS for the determination of sulphonamides in meat [12] and milk [13]. The simultaneous measurement of the N^4-acetyl metabolites of sulphonamides and the parent drugs in swine tissue has been largely ignored [14,15]. Nouws et al. [16] have reported on the residue aspects of the pharmacokinetics of sulphadimidine and its N^4-acetyl metabolites in pigs. In this paper, we describe a procedure for the simultaneous determination of four sulphonamides and their N^4-acetyl metabolites by LC–thermospray-MS and measure its limit of quantification (LOQ) and limit of detection (LOD) by statistical procedures. We also compare the results with a LC-UV method employed in the Bundesgesundheitsamt laboratory.

2. Experimental

2.1. Standards and chemicals

Sulphadiazine (SDZ No. S-8626), sulphathiazole (STH No. S-9876), sulphamerazine (SMZ No. S-8876) and sulphadimidine (SDI No. S-6256) were obtained from Sigma (Deisenhofen, Germany). The N^4-acetyl derivatives of sulphadiazine (NASDZ), sulphathiazole

(NASTZ), sulphamerazine (NASMZ) and sulphadimidine (NASDI) were obtained from Serva (Heidelberg, Germany, No. 39196). [^{13}C]Sulphadimidine ([^{13}C]SDI) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA) and benzthiazurone (BTZ) from Dr. Ehrenstorfer Co. (Augsburg, Germany). Polysulpha-Complex (Bela-Pharm, Vechta, Germany) was used for the treatment of pigs.

Ammonium acetate was obtained from Merck (Darmstadt, Germany, No. 1116). HPLC-grade distilled water was purchased from Promochem (Wesel, Germany). Other reagents and solvents were of analytical-reagent or HPLC grade. Sep-Pak silica cartridges were purchased from Waters Chromatography, Division of Millipore (Milford, MA, USA, No. 51900). Stock standard solutions (0.5 mg/ml) of each sulphonamide and its N^4-acetyl derivative were prepared in acetonitrile. The internal standard benzthiazurone (0.5 mg/ml) and [^{13}C]sulphadimidine (1 mg/ml) were prepared in acetonitrile and ethanol respectively. The stock standards were stored at +4°C and were stable for several months. Working standards were subsequently prepared daily from the stock standards (10 μg/ml) in the LC–MS mobile phase.

2.2. Biological samples

Known sulphonamide-free samples of muscle were collected from animals fattened on a farm owned and maintained by the Institute of Veterinary Medicine of Bundesgesundheitsamt (Berlin, Germany). Incurred material was obtained by intramuscularly injecting nine pigs (5–6 months old) on either side of the neck with the veterinary drug Polysulpha-Complex, containing 8.63 g sulphadimidine-Na, 8.70 g sulphadiazine-Na, 10.83 g sulphamerazine-Na and 4.77 g sulphathiazole-Na (20 ml/100 kg body weight). The animals were slaughtered after 12, 24 or 60 h.

After slaughtering, the carcasses were stored overnight at +4°C and muscle samples were subsequently collected and cut. The samples were packed in aluminium foil as 100-g lots and stored until analysis at −80°C.

2.3. Instrumentation

LC-UV system

The LC-UV system (Waters Chromatography) consisted of two pumps (Model 510), a WISP autosampler (Model 712), a data system and gradient former (Model 810), a temperature control module (40°C), an UV detector with mercury lamp (wavelength 254 nm, Model 440), and a photodiode-array detector (Model 990). The LC column was a Resolve C_{18} column (15 cm × 3.9 mm I.D., 5 μm, Waters). Eluent A was 66 m*M* phosphate buffer (pH 4.6)–acetonitrile (95:5, v/v). Eluent B was acetonitrile–distilled water (80:20, v/v).

Both eluents were degassed and filtered under vacuum through a 0.45-μm filter using a solvent filtration system (Millipore, Milford, MA, USA). The mobile phase was pumped at a flow-rate of 0.8 ml/min. Sulphonamides were separated with gradient elution (0–18 min: 10%; 18–26 min: 15%; 26–35 min: 20%; 35–42 min: 15%; 42–48 min: 10% of eluent B).

LC–MS system

The LC–MS system comprised a pump (Model 590) and an autosampler (WISP, Model 712, Waters). The LC column was a Resolve C_{18} column (15 cm × 3.9 mm I.D., 5 μm, Waters) with a Guard-Pak Resolve C_{18} precolumn. This system was directly coupled to a Vestec Model 201A LC–thermospray-MS system (Houston, TX, USA) equipped with a Teknivent interface and workstation. The mobile phase was acetonitrile–0.1 *M* ammonium acetate (pH 4.6) (13:87, v/v). It was degassed and filtered under vacuum through a 0.45-μm filter using a solvent filtration system (Millipore, Waters). The mobile phase was pumped at a flow-rate of 1.0 ml/min.

The instrument was operated in the positive chemical-ionisation (CI) mode using discharge-assisted ionisation. The electron multiplier voltage was 2200–2600 V. The temperatures of the source block, tip heater and lens assembly were 340, 320 and 170°C, respectively. The vaporizer probe was operated at ca. 10°C below the take-off point. This was optimized for each probe used, but was typically in the region of 200–

230°C. The instrument was operated either in the full-scan mode to collect spectra, or in the selected-ion monitoring mode (SIM) for maximum sensitivity. For the former, a dwell time of 3 ms was used and for the latter, the dwell time was 100 ms. In each case the sweep window was set at 0.5 amu. The tuning parameters were checked daily according the manufacturers instruction using a 50 mg/l solution of polyethylene glycol 300 and adenosine ($M+H=268.2$) in mobile phase.

2.4. Tissue extraction

The procedure developed for the extraction and clean-up of the four sulphonamides and their N^4-acetyl metabolites from muscle samples was similar for LC–MS and LC-UV. However, the internal standard employed was different. For LC–MS analysis samples were spiked with [^{13}C]sulphadimidine at either 200 μg/kg or 40 μg/kg, to permit quantification of either the parent drugs or the N^4-acetyl metabolites, respectively. For the LC-UV procedure benzthiazurone at a concentration of 200 μg/kg was employed as internal standard.

A series of negative and fortified samples were prepared in the concentration range 0–800 μg/kg for each sulphonamide and its N^4-acetyl metabolite. The tissue (5 g) was weighed into a centrifuge tube and blended with chloroform–acetone (15 ml; 2:1, v/v) for 30 s using an Ultra-Turrax blender. After centrifugation at 4000 *g* for 10 min the organic phase was separated and the procedure repeated. The organic extracts were combined and evaporated to dryness using a Speed-Vac concentrator. Dry residues were then dissolved in dichloromethane–hexane (2 ml, 2:1, v/v) and acetone (0.2 ml) with vortex-mixing and ultrasonication (5 min).

2.5. Solid-phase clean-up

Sep-Pak silica cartridges were preconditioned by washing with acetone (5 ml) followed by dichloromethane–hexane (5 ml, 2:1, v/v). After application of the tissue extracts the cartridges were washed with hexane (2×5 ml). The sulphonamides and N^4-acetyl metabolites were eluted with dichloromethane–acetone (2×4 ml, 1:1, v/v). The eluate was concentrated to dryness in a Speed-Vac concentrator at 40°C. Dry residues were dissolved in 300 μl of LC–MS mobile phase with vortex-mixing and ultrasonication. If necessary the samples were filtered through a 0.45-μm filter and placed into a 300-μl microvial. The injection volumes employed for LC–MS and LC-UV were 30 μl and 20 μl, respectively.

3. Results and discussion

LC-UV analyses

The sulphonamides and their N^4-acetyl metabolites could be resolved by LC-UV following off-line solid-phase clean-up as shown in Fig 2. Quantification of each compound was performed with benzthiazurone as the internal standard. With the gradient used it is possible to separate a wide range of sulphonamides, as shown by Malisch [5]. All samples contained [^{13}C]SDI as internal standard for LC–MS as well, which has the same retention time as SDI. Calibration graphs were constructed from duplicate results of negative muscle samples fortified with each sulphonamide and its N^4-acetyl metabolite. Table 1 shows the retention times, the recoveries of the LC-UV method, calculated in the concentration range 50–800 μg/kg, and the coefficients of variation (C.V.), respectively. Recoveries higher than 100% probably result from interfering matrix peaks, which may coelute with some N^4-acetyl sulphonamides (e.g. NASDZ). Least-squares linear regression analysis of the data was performed in the concentration range of 20–100 μg/kg ($n=10$) and the r values (coefficient of correlation) were mainly over 0.96.

LC–MS analyses

Because LC–MS methods provide increased specificity over LC-UV methods of analyses this approach was applied to the determination of the sulphonamides and their N^4-acetyl metabolites. The pH of the ammonium acetate buffer was set to 4.6 to prevent ionisation of the sulphonamides

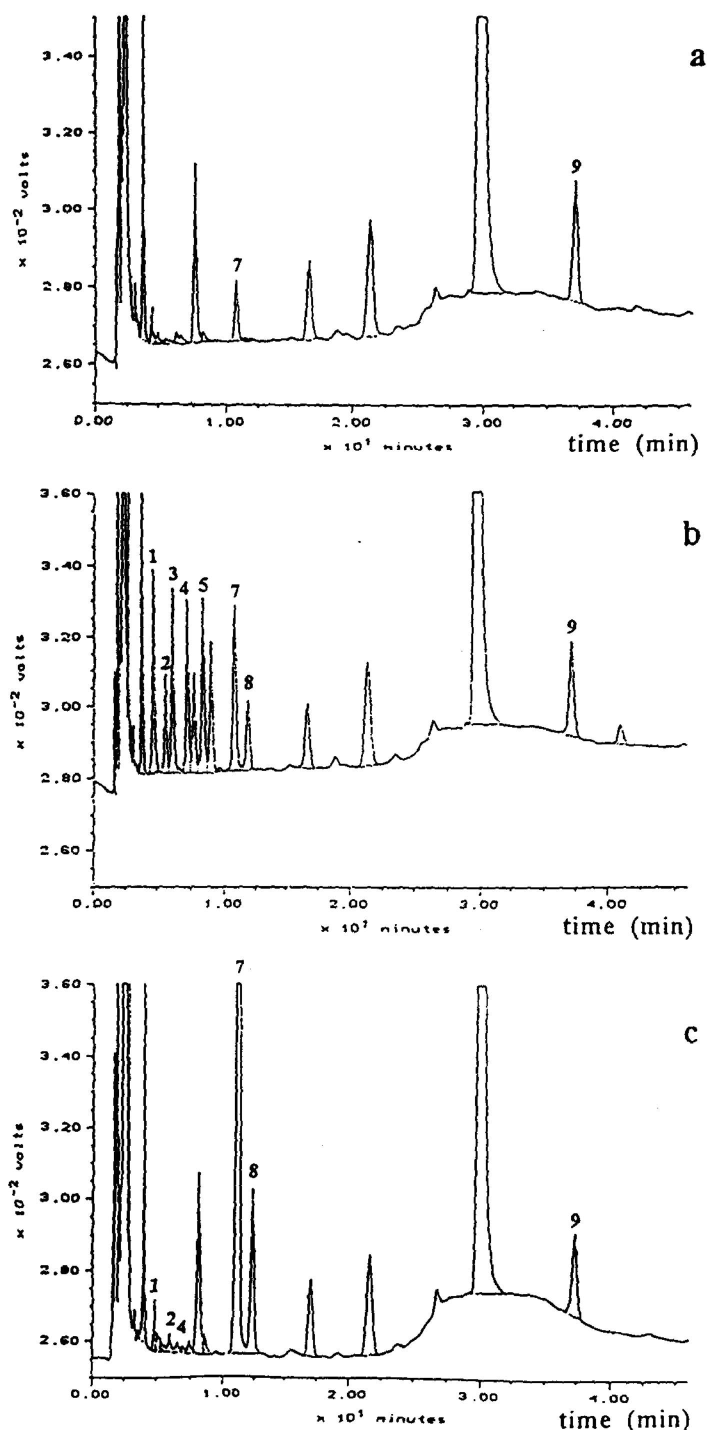

Fig. 2. LC-UV chromatograms of sulphonamides and their N^4-acetyl metabolites. Blank tissue spiked with internal standard (a), blank muscle tissue spiked with 100 μg/kg of standard mixture and internal standard (b), incurred tissue sample and internal standard (c). Internal standard = 200 μg/kg benzthiazurone. Identification of peaks: SDZ (1), STH (2), NASDZ (3), SMZ (4), NASMZ (5), SDI (7), NASDI (8), ISTD (9).

Table 1
Retention times and recoveries of sulphonamides with LC-UV

Sulphonamide	t_R ($n=22$) (min)	C.V. (%)	Recovery (%) ($n=18$)	C.V. (%)
SDZ	4.64	0.30	53.8	14
STH	5.49	0.28	36.0	7
NASDZ	6.02	0.24	142.7	8
SMZ	7.10	0.33	76.1	12
NASMZ	8.25	0.28	108.8	24
NASTH	8.90	0.30	81.1	15
SDI	10.73	0.20	53.0	14
NASDI	11.82	0.23	78.0	8
BTZ (I.S.)	37.07	0.13	82.1	12

during separation on the reversed-phase column. Full-scan spectra could easily be obtained with only 100 ng of each sulphonamide and its N^4-acetyl metabolite. Spectra were obtained in the positive mode for thermospray, filament-assisted ionization and discharge-assisted ionization. In all three cases the full-scan mass spectra were simple and consisted of the protonated molecular ion $[M+H]^+$ as a base peak and very little, if any, fragment-ion signals. The use of positive discharge-assisted ionization gave maximum ion abundances and was therefore employed for SIM. The relevant $[M+H]^+$ ions monitored are listed in Table 2. As the sulphonamides and their N^4-acetyl metabolites have different relative molecular masses the use of a gradient LC regime to prevent co-elution was not necessary. A typical set of single-ion chromatograms for drug-free muscle tissue spiked with a mixed standard at 60 ppb is shown in Fig. 3. The chromatograms were clean and free from interferences from other compounds. Ion chromatograms of incurred muscle tissue are shown in Fig. 4.

Drug-free muscle tissue was spiked in duplicate ($n=10$) with standard solutions of the sulphonamides in the range 50–800 μg/kg together with [^{13}C]SDI internal standard at a concentration of 200 μg/kg and in the concentration range of 20–100 μg/kg with [^{13}C]SDI internal standard at a concentration of 40 μg/kg. Linear regression analysis of the ratio of the area of the [^{13}C]SDI peak to that of the sulphonamide peak was performed and the r values of the calibration curves over the concentration range 50–800 μg/kg were mainly higher than 0.99 (except for SMZ, $r=0.969$ and for NASTH,

Table 2
Limits of detection and limits of quantification by LC–MS compared with LC-UV

Sulphonamide	LC-UV		LC–MS		Ion (m/z)
	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	
STH	9.2	113	22	124	256
SDZ	6.3	76	5.3	86	251
SMZ	2.3	60	2.5	88	265
SDI	9.3	61	2.6	44	279
NASTH	4.6	66	23	125	298
NASDZ	3.0	66	5.6	81	293
NASMZ	3.3	64	3.1	74	307
NASDI	7.7	64	4.4	107	321
[^{13}C]SDI	–	–	–	–	285

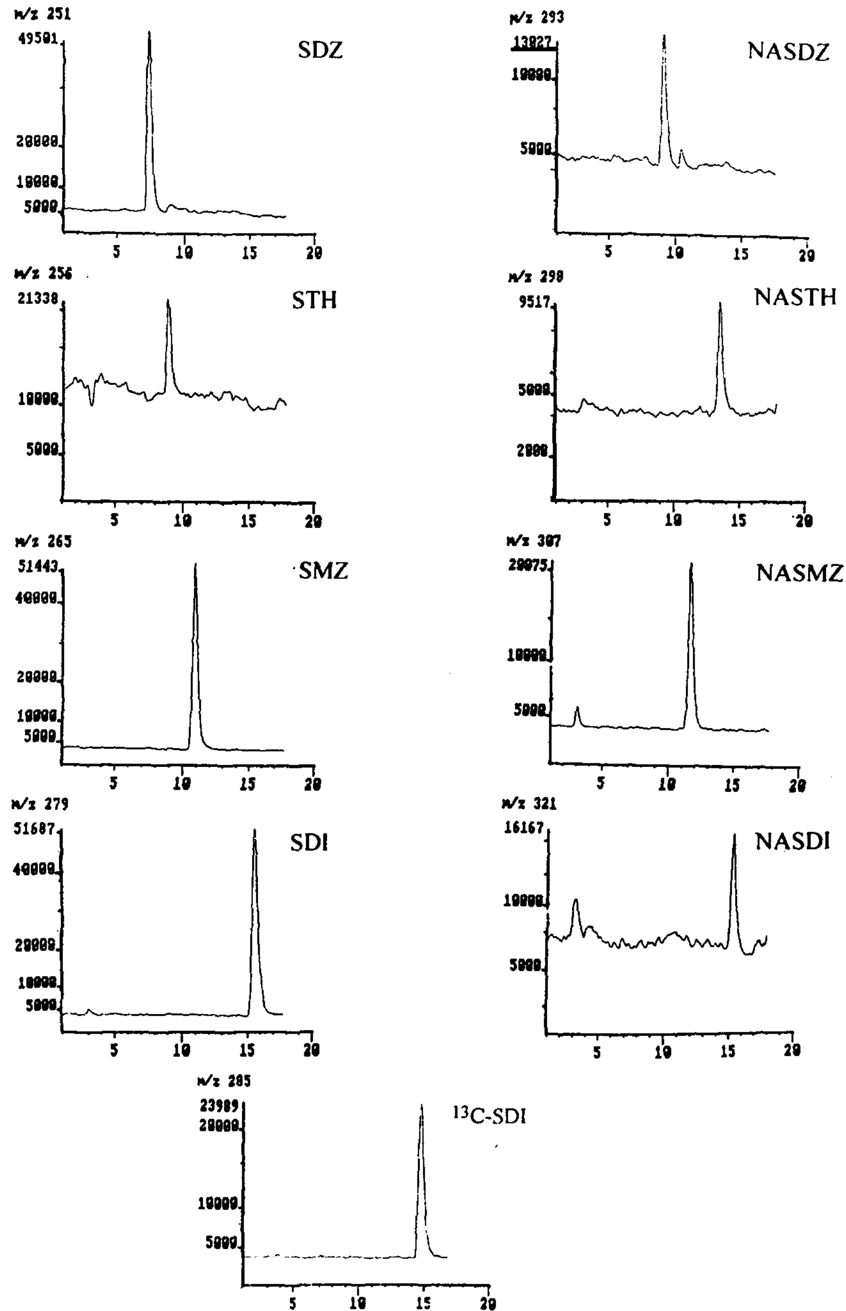

Fig. 3. Single-ion chromatograms: muscle sample spiked with 60 μg/kg sulphonamides, N^4-acetyl sulphonamides and the internal standard 40 μg/kg ([^{13}C]SDI).

$r = 0.989$). In the concentration range 20–100 μg/kg the r values were 0.90–0.98 (except for NASTH).

Limits of detection (LOD) for the LC–MS method were calculated using representative blank swine muscle tissue ($n = 22$). The LOD

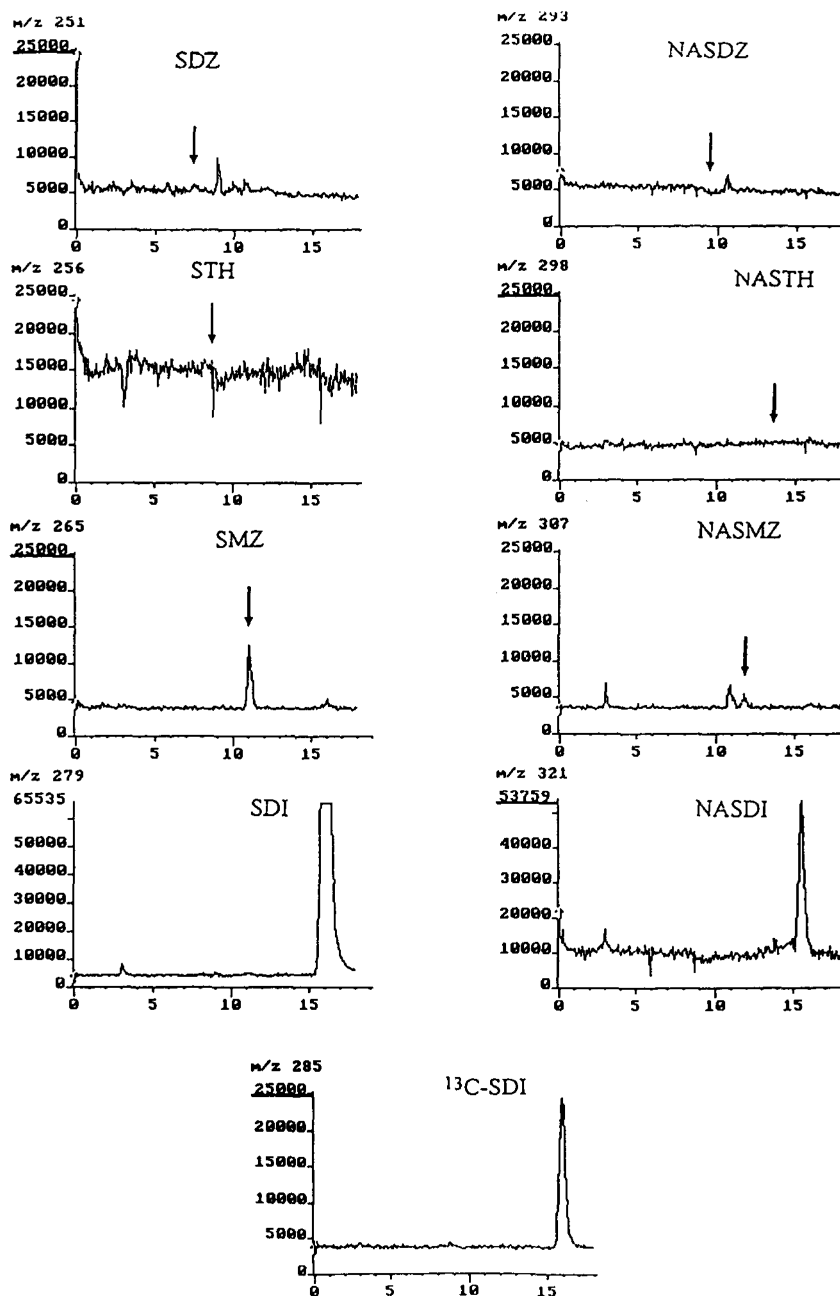

Fig. 4. Single-ion chromatograms of incurred tissue and the internal standard 40 μg/kg ([^{13}C]SDI).

values calculated from the standard deviation (S.D.) of the mean noise for a blank tissue sample (LOD = mean + 3 × S.D.) are shown in Table 2. LOD values for the LC-UV method were calculated, based on a signal-to-noise ratio of 3:1 for spiked samples ($n = 5$).

In the assay described in this paper the limits of quantification (LOQ) were calculated from

Table 3
Comparison of assay results for the level of sulphadimidine detected in incurred muscle tissue by a range of LC-UV and LC–MS methods ($n = 2$–7)

Method	Year	Sulphadimidine (μg/kg)	C.V. (%)	N^4-acetyl-sulphadimidine (μg/kg)	C.V. (%)
LC-UV	1988/89	981	16	n.a.	
LC-UV	1992/93	1256[a]	8.5	184	8.8
LC–MS	1992	1003	9.8	181	6.4
LC–MS	1993	990	9.7	178	4.5

n.a. = not analysed.
[a] Concentration is outside the calibration range.

spiked muscle tissue (20–100 μg/kg, $n = 10$) using the method of Funk et al. [17]. The results are shown in Table 2.

The absolute signals obtained for the LC–MS method were consistent during a run but were variable from day to day. This may result from alteration of the spray characteristics of the probe caused perhaps by contamination. This was overcome by use of an internal standard ([^{13}C]SDI).

The inter-day precision of the LC–MS assay has coefficients of variation (C.V.) of 2–27% for all substances, calculated at the 80–100 μg/kg concentration level. The reproducibility of the assay was determined by analysing a muscle sample obtained from a pig treated with Poly-sulpha-Complex drug in 1987 and stored at −80°C. Only sulphadimidine and its N^4-acetyl metabolite were detected (SMZ residues were detected below the LOQ level). The results are shown in Table 3.

The range of concentrations of SDI and NASDI present in this sample required their separate measurement, employing an appropriate amount of internal standard. The incurred muscle sample was analysed on a previous occasion (1988/89), by a different LC-UV method, developed in the Bundesgesundheitsamt, and the results are shown in Table 3. As previously, SDI was the only sulphonamide found. Due to inhomogeneity or instability of the cut-and-packed meat samples some changes in the concentration of the analytes over the years of storage can be observed. It is recommended that measurements outside the validated calibration range should be avoided and highly concentrated samples be diluted to within this range for quantification.

4. Conclusions

The described LC–MS and LC-UV assays are reproducible and the LOQ of all compounds, except those of sulphathiazole and its N^4-acetyl metabolite, met EEC regulations which permit a maximum residue level (MRL) of 100 μg/kg sulphonamides in edible tissues. The LC–MS method has sufficient specificity to differentiate between several sulphonamides and their N^4-acetyl metabolites.

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