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Note

Identification of a novel side-product formed during the methylation of sulphapyridine prior to gas chromatographic analysis

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The monitoring of sulphonamide residues in edible animal tissues is carried out in the U.K. as part of a food safety surveillance programme, and elsewhere routinely for regulatory purposes. Quantitative analytical procedures have been fully developed and commonly involve extraction, concentration and clean-up from the tissue prior to derivatization and gas chromatographic (GC) analysis with flame ionization detection[1], electron-capture detection[2] or mass spectrometric (MS) selected ion monitoring[3–6]. The most frequently described derivatization procedures for sulphonamides involve either a single stage N^1-methylation with diazomethane[1,5,6] (methylation of the nitrogen adjacent to the SO_2), or N^1-methylation followed by acylation of the NH_2 function with pentafluoropropionic anhydride or other appropriate reagents[3,4]. These derivatization procedures have been widely adopted for the whole range of sulphonamide drugs, and in conjunction with their stable isotope analogues have been applied in quantitative GC–MS procedures[3–6].

During the course of method development for sulphonamide residue analysis, we have checked the methylation of a number of standards by direct insertion probe MS. In addition to the anticipated N^1-methyl derivatives for which spectra were obtained which were in good agreement with the published literature, in a number of instances fractionation occurred from the probe and an additional minor isomeric product was observed. This paper reports the isolation, purification by high-performance liquid chromatography (HPLC) and identification by nuclear magnetic resonance (NMR) spectrometry and MS of the side product formed during the methylation of the sulphonamide, sulphapyridine.

EXPERIMENTAL

Materials

Sulphapyridine was obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.) and 1-methyl-3-nitro-1-nitroguanidine from Aldrich (Dorset, U.K.)

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Derivatization

An aliquot of the sulphapyridine (or other sulphonamide standard) dissolved in methanol was evaporated to dryness, redissolved in 100 μl of methanol and methylated by treatment with 0.5 ml of a saturated solution of diazomethane in diethyl ether (prepared from 1-methyl-3-nitro-1-nitrosoguanidine using a diazomethane generation apparatus)[7]. After allowing to stand for 30 min for reaction, the solution was evaporated to dryness and taken up in the appropriate solvents for subsequent analysis.

Mass spectrometry

Electron ionisation mass spectra were obtained on a VG 12000 quadrupole mass spectrometer using direct insertion probe introduction, allowing the sample to vaporise by indirect heating from the MS source held at 200°C. Ionisation was at 70 eV, with the trap current at 200 μA. The mass spectrometer was repetitively scanned from m/z 33 to 600 in 1 s and the spectra were acquired and processed with a VG DS2000 data system.

GC–MS was carried out with a Carlo Erba 4160 GC instrument directly coupled to the above mass spectrometer, using a 25 m × 0.23 mm I.D. fused-silica CP SIL7 column (Chrompack, U.K.) operated isothermally at 270°C with helium carrier gas at 0.8 bar. Injection of samples (1.5 μl) was in a split mode (20:1) with the injector temperature set at 280°C.

HPLC isolation of the minor methylation product

The HPLC system consisted of a Waters Model 660 controller and two Model 6000A pumps (Millipore, Bedford, MA, U.S.A.), a Rheodyne 7125 injection valve with a 20-μl loop and a Pye LC-UV detector fitted with an 8-μl flowcell and set at 254 nm. The column (Spherisorb ODS, 5 μm 250 × 4.9 mm I.D.) was operated at a solvent flow-rate of 1 ml/min with a mobile phase of methanol in water programmed linearity over 15 min from 0% to 100% methanol. Total loading of the column was about 20 μg of the methylated mixture per run, and trapping was at the exit of the UV detector collecting the desired component in about 1.5 ml of solvent.

NMR analysis

The purified components isolated by HPLC were evaporated to dryness and redissolved in [$^2H_6$]dimethylsulphoxide. Proton NMR spectra were recorded at 90 MHz on a Perkin Elmer R32 NMR spectrometer, shifts being reported in parts per million (ppm) relative to tetramethylsilane internal standard. Carbon-13 NMR spectra were obtained at 50 MHz on a Nicolet NTC FT-NMR spectrometer.

RESULTS AND DISCUSSION

The electron impact mass spectra of both the expected N^1-methylation product and the additional minor component are shown in Fig. 1. Both spectra could be separately obtained by fractionation of the derivatization products on the MS probe. Confirmation of the success of the trapping operation from the HPLC was demonstrated by obtaining identical spectra to those in Fig. 1 for the individual isolated components. GC–MS analysis showed the N^1-methyl derivative at a retention time

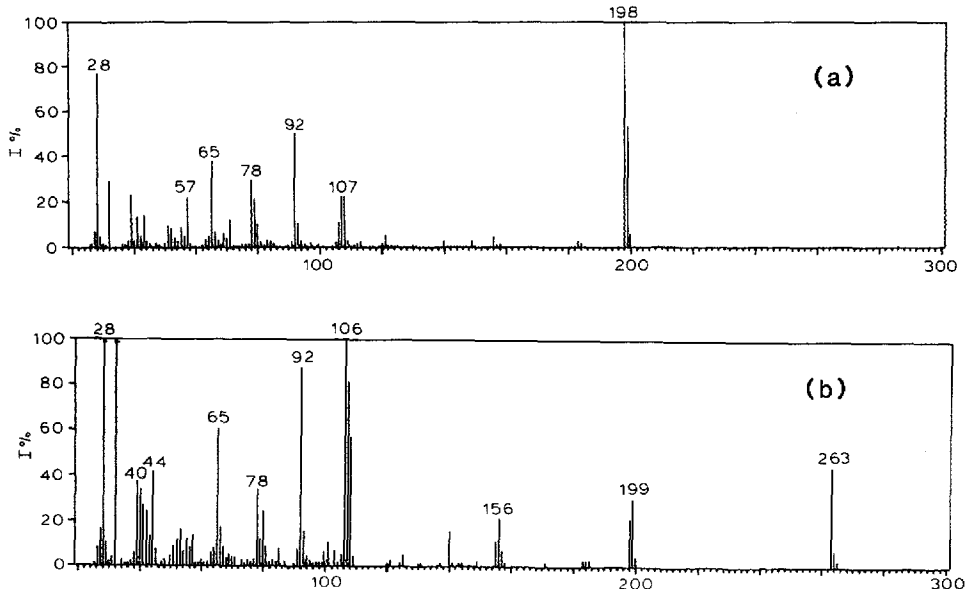


Fig. 1. Probe electron impact mass spectra of (a) ^{1}N-methylated sulphapyridine (MW = 263) and (b) sulphapyridine methylated on the pyridine ring nitrogen (MW = 263). Spectra obtained by fractionation from the heated probe.

of 8.75 min with an unchanged mass spectrum from that obtained by probe analysis. The other product could not be detected by GC–MS, and was therefore presumed to be too polar to be chromatographed under these conditions of analysis.

A typical HPLC chromatogram is given in Fig. 2, showing the underivatized sulphapyridine at a retention time of 13.6 min, the minor methylation product at

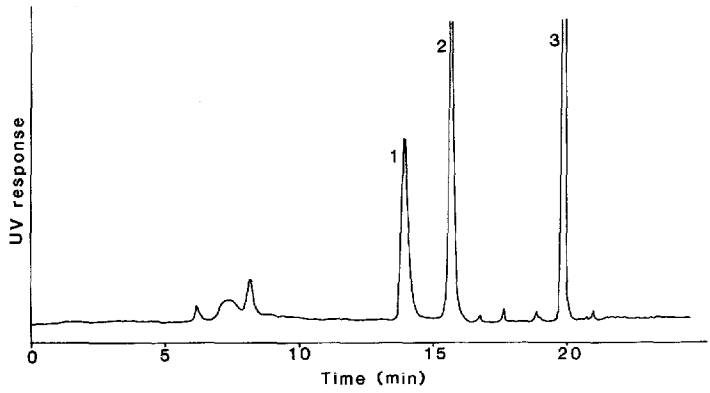
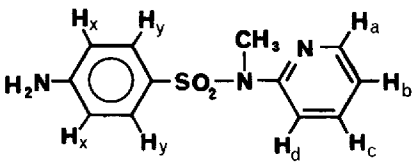


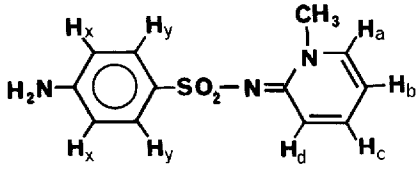
Fig. 2. HPLC chromatogram illustrating the separation of (1) underivatized sulphapyridine, (2) derivative methylated on the pyridine ring nitrogen and (3) derivative methylated on the sulphonamide nitrogen adjacent to the SO_2. HPLC conditions: Spherisorb ODS column operated at a flow-rate of 1 ml/min with a mobile phase of methanol in water programmed from 0 to 100% over 15 min. UV detection at 254 nm.

TABLE I

PROTON NMR ASSIGNMENTS FOR METHYLATED SULPHAPYRIDINE DERIVATIVES



I

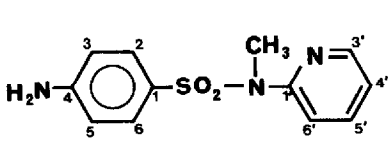


II

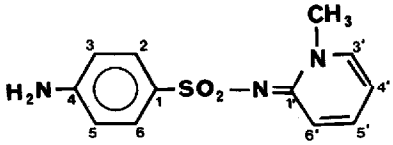
Proton	*Shift relative to TMS (ppm)*	
	I	*II*
H_a	8.28	8.04
H_b	7.17	6.63
H_c	7.80	7.65
H_d	7.56	7.35
H_x	6.53	6.55
H_y	7.17	7.46
N–H	6.07	5.70
$N–CH_3$	3.13	3.65

TABLE II

CARBON-13 NMR ASSIGNMENTS FOR METHYLATED SULPHAPYRIDINE DERIVATIVES



I



II

Carbon	*Shift relative to TMS (ppm)*	
	I	*II*
C-1	130.0	124.3*
C-2 and C-6	122.9	127.7
C-3 and C-5	113.8	112.6
C-4	153.8	151.5
C-1′	154.2	**
C-3′	148.6	141.3
C-4′	120.3	116.1
C-5′	138.9	110.3
C-6′	122.0	140.5
$C(CH_3)$	36.1	40.9

* Signal very weak.
** Signal not observed.

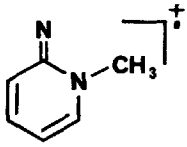
15.3 min and the N^1-methyl derivative at 19.5 min. These HPLC retention times indicate the product of intererest to be considerably more polar than the N^1-methyl derivative, but slightly less polar than the underivatized sulphapyridine.

The proton and carbon-13 NMR results are given in Tables I and II, respectively, both for the minor methylation product (compound II) and for comparative purposes for the N^1-methyl derivative (compound I). It can be seen from the assignments that the minor product is proposed as having a structure with methylation having occurred on the pyridine ring nitrogen. It is apparent in the proton spectra for the two compounds that H_x and H_y protons in both cases occur at essentially the same shift values, indicating that in the unknown substitution at or near the aniline ring is unlikely. All shift values for protons H_{a-d} are upfield of those of the N^1-methyl derivative, consistent with a reduction of aromatic character in the pyridine ring. Also consistent with this loss of aromatic character is the observed increase in nearest neighbour coupling constants (similar to those found in substituted butadienes) and the loss of long range couplings observed in aromatic pyridine systems.

Similarly for the ^{13}C-NMR spectra the shift values for the aniline ring carbons for the two sulphonamides are essentially the same, and the significant change in shift value is only observed for the pyridine ring of the unknown. The CH_3 resonances occurring at 36.1 for the N^1-methyl derivative and at 40.9 for the unknown are in both cases consistent with N–CH_3 linkage rather than an O–CH_3 moiety.

The NMR data alone do not conclusively demonstrate the methylation of the pyridine ring nitrogen in the unknown, but this characterization is further supported by the MS data. The mass spectrum of the N^1-methyl derivative (Fig. 1) was consistent with published data[8] with the molecular ion absent, and the presence of intense ions at *m/z* 198 and *m/z* 199 through the characteristic losses of HSO_2 and SO_2, respectively. In contrast, and unusually for a sulphonamide, the unknown shows a molecular ion at *m/z* 263 (48%), and although ions are present indicating loss of HSO_2 and SO_2, this is clearly not as facile a loss as for most sulphonamides. It is proposed that the retention of SO_2 and stabilisation of the molecular ion is due to extended conjugation into the methylated pyridine ring system:

Also of note in the mass spectrum of the unknown is the base peak at *m/z* 106 and it is suggested that this has the following structure:



again supporting the proposed methylation of the pyridine ring nitrogen. Infrared analysis of the product showed evidence for N–H, S=O and a signal at 1650–1740 cm^{-1} that could be interpreted as a C=N stretch.

CONCLUSION

The combined spectroscopic data support the supposition that during diazomethane methylation of sulphapyridine although the major product is the N^1-methyl derivative, a minor mono-methylated product is also formed with methylation of the pyridine ring nitrogen. This unusual methylation can be explained mechanistically on the basis of initial proton abstraction from sulphapyridine by diazomethane (the accepted mechanism for diazomethane methylation), with rearrangement atypically then occurring of the anion, followed finally by the accepted route of attack on the diazomethane cation:

In the methylation of other sulphonamides, *e.g.* sulphamethazine and sulphadiazine, analogous isomeric methyl derivatives were observed to be formed as minor products at levels of a few percent of that of the corresponding N^1-methyl derivatives. The greatest amount of the product occurred with sulphapyridine at levels of about 5%. These secondary derivatization products are of some interest in that they should be recognised as representing a potential minor source of error in the quantitative analysis of sulphonamides.

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