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Bound sulphadimidine residues in raw fermented sausage: release under acidic conditions and bioavailability in rats

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Abstract Sulphadimidine (SDM), a drug frequently administered to pigs, is partially converted into other compounds by processing meat to produce raw, fermented sausage. With the aid of ^{14}C-labelled SDM, evidence was obtained that part of the radioactive matter was covalently bound to the matrix. Part of these bound residues could be released in vitro by 4 M HCl at 21 °C or by 0.024 M HCl at 37 °C. Female rats were also able to release bound SDM residues and to excrete these in their urine, in amounts approaching those obtained by treatment with 4 M HCl. Both the parent compound and its main metabolite, N^4-acetyl-SDM, were observed in the urine of rats.

Key words Sulphadimidine · Fermented sausage · Carbon-14-labelling · Rat · Bound residues

Introduction

Residues of veterinary drugs in meat and meat products can be classified in four categories: (1) the parent drug, (2) metabolites produced by the animal, (3) products resulting from non-physiological chemical reactions, and (4) residues bound to the matrix. This last category is defined as compounds covalently bound to macromolecules. These bound residues cannot be extracted by aqueous solutions or organic solvents.

With regard to veterinary drugs, non-extractable residues of the following have been observed: furazolidone in swine tissues [1]; cambendazole in sheep liver [2]; trenbolone acetate in cattle tissues [3]; ronidazole in swine muscle [4–6]; toluoyl chloride phenyl hydrazone (TCPH) in sheep blood [7]; and sulfadimidine (SDM), after reaction with nitrite in the gastro-intestinal tract, in swine [8] and in rats [9].

Covalently bound residues can be released in vitro by oxidation, reduction, through the action of proteolytic enzymes, or by acid hydrolysis, depending on the compound under consideration. The release of covalently bound residues by these methods has been observed for TCPH [7], cambendazole [2], trenbolone [3], ronidazole [4] and furazolidone [10]. Their in vivo release has also been studied, mainly in rats, using radiolabelled compounds. For cambendazole [2], TCPH [7] and SDM in the presence of nitrite [9], only a small proportion of the bound residues could be released. For ronidazole [5] and furazolidone [11], about half of the radioactivity could be recovered from urine or from the carcass.

Bound residues can also be formed during processing of meat, e.g. from penicillin during the cooking of meat [12] and from SDM during the preparation of raw, fermented sausage [13, 14]. With respect to SDM, up to 20% of the original amount of ^{14}C-SDM was neither extractable with dichloromethane, nor by extraction with phosphate buffer and, subsequently, could not be extracted with methanol [14]. Extraction by chloroform-acetone, followed by phosphate buffer and by methanol, could not release this proportion of SDM either [14]. Furthermore, ^{14}C-SDM could not be extracted with phosphate buffer nor, subsequently, by dichloromethane and methanol (Smit, unpublished results).

The objective of the present study was to investigate whether covalently bound residues generated from SDM during the preparation of raw fermented sausage, could be released in vitro and in vivo. A preliminary experiment showed the presence of unbound residues (Smit, unpublished results). The pre-extraction used, however, is unsuitable under acidic conditions, leaving too many matrix components in the extract to avoid interference with respect to the determination of SDM. For this reason, another pre-extraction procedure was

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applied, i.e. extraction with methanol, followed by ethanol and diethyl ether.

Covalently bound residues in the sausage residues were successively subjected to in vitro or in vivo methods of release. In vitro release was studied under strongly acidic (4 M HCl) conditions. To simulate acidic conditions occurring in the stomach, release in 0.024 M HCl was studied as well. The in vivo release of SDM (bioavailability) was studied in rats. Analysis was performed by using solid–phase extraction (SPE) and HPLC, followed by UV detection.

Materials and methods

Materials and chemicals

SDM was obtained from Sigma (St. Louis, Mo.). N^4-acetyl–SDM (N4AS) was synthesised as described by Nielson [15]. ^{14}C-SDM (specific activity 14.4 MBq mg^{-1}) was obtained from Amersham (Buckinghamshire, UK) and was labelled in the benzene ring. Purification by HPLC was performed before use. Methanol, diethyl ether and acetonitrile were obtained from Rathburn (Walkerburn, UK) and were HPLC grade. The other chemicals and ethanol were obtained from Merck (Darmstadt, Germany). Alumina was obtained from Janssen (Beerse, Belgium), and silica-bonded phenyl SPE columns (0.5 g/3 ml–1) from Baker (Phillipsburg, N. J.). Disposable alumina extraction columns were prepared by filling 3-ml columns (Baker) with 2 g alumina, and were packed with a fritted disc over the sorbent bed. S&S 589.1 filters (diameter 110 mm) were obtained from Schleicher and Schüll (Dassel, Germany), and the destruction fluid, Soluene-350, and the scintillation cocktail, Dimilume-30, from Packard (Delft, The Netherlands).

Meat was purchased from a local slaughterhouse, and the additives were obtained from Verstegen (Rotterdam).

Apparatus

The following apparatus were used: a Vortex mixer (Scientific Industries, Bohemia, Florida, USA); a Moulinette homogeniser (Moulinex, Gouda, the Netherlands); a Bransonic B221 ultrasonic bath (50–60 kHz), with tray insert (Branson Europe, Soest, The Netherlands), filled with water; an HPLC pump (Pharmacia/LKB 2150, Uppsala, Sweden); a Rheodyne 7125 injector (Cotati, Calif.), equipped with a 50-µl loop; an Applied Biosystems 783A detector (Maarssen, The Netherlands), operated at 254 nm and connected to a SP4270 integrator (Spectra-Physics, San Jose, Calif.); and a PW 4700 liquid scintillation counter (Philips, Eindhoven, The Netherlands). The counting efficiency was calculated by means of an external standard counting procedure.

Experimental design

For the release studies, three dough batches were prepared, i.e. two batches containing SDM at initial levels of 10 mg kg^{-1} and 1 mg kg^{-1}, respectively, and a blank. These batches were used for sausage preparation as described before [14]. After 48 h in brine, and ripening for 4 weeks, the sausages were examined. The casings were removed, the sausages were homogenised, and 10–g portions were accurately weighed in a 100-ml beaker. All samples were pre-extracted as described below.

For the in vitro release of covalently bound SDM residues, the residues of the extracted sausage samples were subjected to the acidic treatments described below. After this, the amount of SDM was determined by SPE, followed by HPLC and UV detection. For the in vivo release, the residues of the samples were administered to rats. Urine was collected and stored at –20 °C until analysis. The amount of SDM and its main metabolite, N4AS were determined as described below.

Pre-extraction. Homogenised sausage (10 g) was weighed in a 100 ml-beaker. A 25-ml volume of methanol was added, and the mixture was stirred with a glass rod. The beaker was placed on the tray insert of the ultrasonic bath for 10 min, the water temperature being kept below 30 °C. The solvent was removed by filtering the mixture through a S&S 589.1 filter paper. This extraction was repeated twice with 25 ml methanol, 3 times with 25 ml ethanol, and twice with 25 ml diethyl ether. The extracts were discarded.

Experiments with radio-labelled SDM. Starting from the result of an earlier study [14], experiments with 14C-SDM were performed to determine the amount of bound residues in the residues of extracted sausage. In the present experiment, sausages were used that were manufactured from dough to which ^{14}C-SDM was added at a rate of 1 or 10 mg kg^{-1} [14]. After treating these with brine and ripening for 4 weeks, the sausages were pre-extracted with methanol, ethanol and ether, as described above. A 0.4 g-portion of the remaining sample was destructed with Soluene-350 and bleached with hydrogen peroxide.

The radioactivity was counted after the addition of Dimilume-30 [14], in a liquid scintillation counter.

In vitro release by 4 M HCl. The residue of a 10-g portion of sausage was dried overnight and transferred to a clean 100-ml beaker. A 50-ml volume of 4 M HCl was added. The mixture was left for 60 min at room temperature (21 °C). After this period, the liquid was filtered using a S&S 589.1 filter paper. The funnel was rinsed twice with 10 ml water. To the combined filtrate, 22 ml of 10 M sodium hydroxide was added. This solution was adjusted to pH 6.5 with phosphoric acid. The clean-up and concentration steps were performed by a phenyl SPE column pre-treated with 3 ml methanol, followed by 3 ml of 0.1 M phosphate buffer at pH 6.5, on a vacuum manifold.

The SPE column was removed from the manifold, dried by compressed air, and eluted with 3 ml methanol by gravity flow. The eluate was reconstituted in 1.0 ml mobile phase. This solution was used for chromatography.

For recovery determination, samples of extracted sausage were spiked with SDM at a rate of 1 mg kg^{-1}, both before and after treatment with 4 M HCl.

In vitro release by 0.024 M HCl. The residue of a 10-g portion of sausage, after extraction, was dried overnight, and transferred to a clean 100-ml beaker. A 50-ml volume of 0.024 M HCl was added, and the beaker was placed in a water bath at 37 °C for 4 h. The liquid was filtered using a Büchner funnel. The funnel was rinsed twice with 10 ml water. To the combined filtrate, 10 ml trichloroacetic acid in water (0.5 g ml^{-1}) was added. Replacement of phosphate by trichloroacetic acid was necessary in order to remove finely dispersed matter in the solution which clogged the clean-up system.

The mixture was centrifuged for 10 min at 2500 g. To the supernatant a solution of 50 ml 30% sodium chloride in water was added and adjusted to pH 6.5 with 5 M sodium hydroxide solution (approximately 6 ml). The resulting liquid, approximately 100 ml, was cleaned up and concentrated using a phenyl SPE column, as described above, with 4 M HCl.

For recovery determination, samples were spiked with SDM at a rate of 1 mg kg^{-1}, both before and after the acid treatment.

In vivo release. Wistar rats (obtained from the Central Animal Laboratory, Utrecht University), each weighing 300 g, were housed individually in metabolism cages, under a 12-h light/dark cycle. Two male and two female rats received four portions of pre-extracted sausage each, originating from 10-g portions of sausage prepared from dough containing 10 mg kg^{-1} SDM. A second group of two male and two female rats received the extracted

blank sausage. Before administration, the rats were deprived of feed overnight, but given water ad libitum. Urine was collected separately from the faeces daily for 3 days, and stored at –20 °C until required for analysis.

The urine was cleaned up and concentrated by the following procedure: 3 ml of 0.05 M phosphate buffer at pH 6.5 was added to 1 ml urine. This solution was passed through a phenyl SPE column which was pre-treated with 3 ml methanol followed by 3 ml of 0.05 M phosphate buffer at pH 6.5. The column was rinsed twice with 3 ml phosphate buffer, and dried in a stream of air for 1 h. Methanol (5 ml) was used to elute the column. The eluate was directly applied to an alumina column (pre-treated with 3 ml water followed by 5 ml methanol). The alumina column was dried in a stream of air for 1 h and eluted with 10 ml water. This eluate was directly applied to another pre-treated phenyl SPE column. After drying in a stream of air, this second phenyl SPE column was eluted with 3 ml methanol. The eluate was collected and reconstituted in 0.5 ml mobile phase. This solution was used for chromatography.

For recovery determination, urine samples were spiked with SDM or N4AS at a rate of 0.4 mg kg^{-1}.

Chromatography. For the in vitro release studies, HPLC was performed using a glass column (100 × 3.0 mm, Chrompack) containing Lichrosorb RP8 (7 μm, Chrompack), connected to a guard column (10 × 2.1 mm) packed with RP8 (Chrompack). The mobile phase was 0.02 M acetate buffer (pH 4.9):methanol:acetonitrile (8:15:4, v/v/v), and the flow rate was 0.5 ml min–1.

For the in vivo release study, two glass HPLC columns (100 × 3.0 mm, Chrompack) were used containing Lichrosorb RP8 (7 μm, Chrompack), connected to a guard column (10 × 2.1 mm) packed with RP8 (Chrompack). The mobile phase was 0.01 M phosphate buffer (pH 6.67):methanol (8:2, v/v), and the flow rate was 0.8 ml min–1. Detection was at 254 nm.

Both systems operated at room temperature. Data were obtained with the integrator. An aliquot (50 μl) of the samples and standard solutions were injected by means of a loop injector.

Results and discussion

For comparison with, unbound SDM was analysed by methods previously described earlier studies [13, 14], showing that 25.1% and 28.3% of the original amount could be extracted from the sausage prepared from dough which initially contained levels of 1 mg SDM kg^{-1} or 10 mg SDM kg^{-1}, respectively.

Radioactivity studies

When N4AS was added to the sausage meat at rates of 1 mg kg^{-1} and 10 mg kg^{-1}, 21.2% and 15.7% of the radioactive matter added remained in the sausage residues, respectively. The result for the 1 mg kg^{-1} rate of N4AS addition was in good agreement with earlier results, while for the 10 mg kg^{-1} rate the results were slightly higher [13, 14].

In vitro release by 4 M HCl

The release of SDM from the sausage residue is given in Table 1 and is also shown in Fig. 1. Calculations were based upon the observation that 100 g dough yields 80 g sausage.

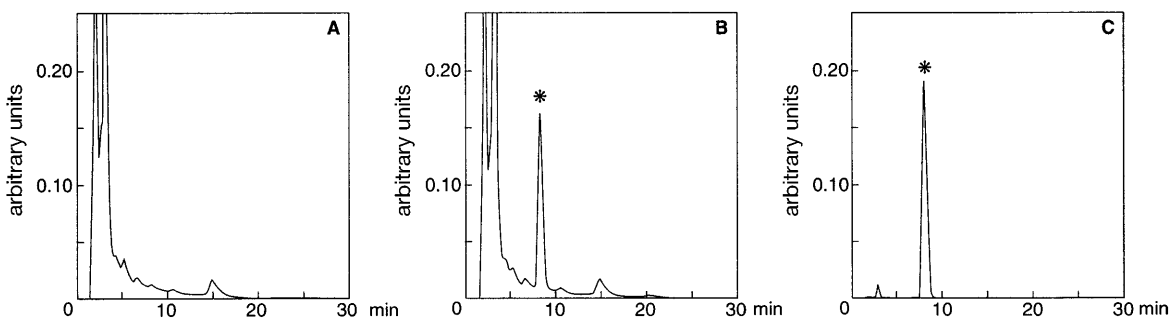
The method for the analysis of SDM in aqueous liquids was the same as that for SDM, SOH and DAS in brine described previously [13]. pH adjustment, however, was performed with an excess of 10 M sodium hydroxide and subsequent drop-wise addition of 0.1 M phosphoric acid.

In order to establish recovery percentages, sausage residues spiked with 1 mg SDM kg^{-1} were extracted with 4 M HCl, and SDM was added either directly after extraction or after 1 h at 21 °C. The percentage recoveries were at an acceptable level [before extraction with

Table 1 In-vitro sulphadimidine (*SDM*) release from spiked sausage samples treated with 4 M HCl, and with 0.024 M HCl, respectively, after pre-extraction

SDM added to dough (mg kg^{-1})	Release of (SDM mg kg^{-1}) calculated on the basis of sausage residue	Release of SDM (%) calculated on the basis of original amount of SDM added
4 M HCl		
1	0.21	17.2
10	1.56	12.6
0.024 M HCl		
1	0.17	14
10	0.8	6

Fig. 1 A–C Chromatograms of sausage residue after in vitro release by 4 M HCl at 20 °C. **A** blank sausage, **B** sausage prepared from dough containing 10 mg sulphadimidine (SDM) kg–1, **C** SDM standard (*)



4 M hydrochloric acid 88.3% (SD = 3.6, $n = 4$); and after extraction, 88.2% (SD = 6.8, $n = 4$)]. Recoveries were thus independent of spiking prior to, or after, acid extraction ($P > 0.9$).

The amount of SDM released did not increase during acidic treatment for longer than 1 h. It was slightly lower than the amount remaining in the radioactivity study. This indicated that a small amount of bound SDM residue is non-extractable, even with 4 M HCl.

Hydroxy-desamino-SDM (SOH), and desamino-SDM (DAS) could not be detected after the acid treatment (data not shown).

In vitro release by 0.024 M HCl

Sausages, after pre-extraction, were subjected to 0.024 M HCl. SDM was partially released (Table 1). Further experiments showed that SDM release under these conditions is slow, and needs at least 4 h of treatment with 0.024 M HCl to reach a maximum.

The determination of SDM in this liquid was the same as that used following treatment with brine [13, 14] with a few modifications, i.e. precipitation of interfering substances was by trichloroacetic acid, and 30% sodium chloride was added to increase the amount recovered.

Recovery percentages following the addition of SDM at a rate of 1 mg kg^{-1} were established before and after extraction with 0.024 M HCl for 4 h at 37 °C; in spite of the measures carried out, recoveries for SDM were low (60% vs. 50% respectively). The difference was small but significant, and may be explained by the slow reactions of SDM with matrix components during these 4 hours. We assumed a 60% rate of recovery in order to estimate the amounts released.

The recoveries of SOH and DAS were in the same order of magnitude as that for SDM. Neither SOH nor DAS could be detected in spiked samples after this release (data not shown).

The results suggested that release by 0.024 M HCl is slightly lower that that by 4 M HCl.

In vivo release in rats

The total amount of pre-extracted sausage administered to each rat was 40 g, which was obtained from 50 g dough containing 0.5 mg SDM.

Results of the in vivo release are shown in Table 2. When rat urine was spiked with SDM and N4AS at a rate of 0.4 mg kg^{-1}, the recoveries were 73.7% (SD = 7.0%, $n = 6$) for SDM and 58.4% (SD = 6.1%, $n = 6$) for N4AS.

The two female rats excreted SDM to a larger extent than the male rats. This lower amount in male rats could be explained by differences in metabolism. In male rats, a considerable proportion of SDM is transformed into 4′-hydroxy-SDM (S4OH) and 5′-hydroxymethyl-SDM (S5OH), while in female rats these metabolites are found in very low amounts [16]. These two metabolites, as a result of interference with matrix compounds, could not be detected at low levels.

In an experiment with sausage made from dough containing 1 mg SDM kg^{-1}, SDM and N4AS in the urine of rats were below detection limits.

One of the degradation products of SDM, DAS, could be detected in the rats' urine. In the sausage residues from spiked dough samples, however, this compound could not be detected. Another degradation

Table 2 Bioavailability of SDM and its metabolite N^4-acetyl-SDM (*N4AS*) in the rat, from sausages previously extracted to remove unbound residues. The amounts released in vitro are given in μg

		SDM	N4AS	Total calculated as SDM (SDM + 0.869 N4AS)	Release (%) calculated on the basis of original amount of SDM added
Male	1	1.1	9.2	9.0	1.8
	2	2.3	5.6	7.2	1.4
Female	1	6.5	54.2	53.5	10.7
	2	9.9	59.6	61.6	12.2

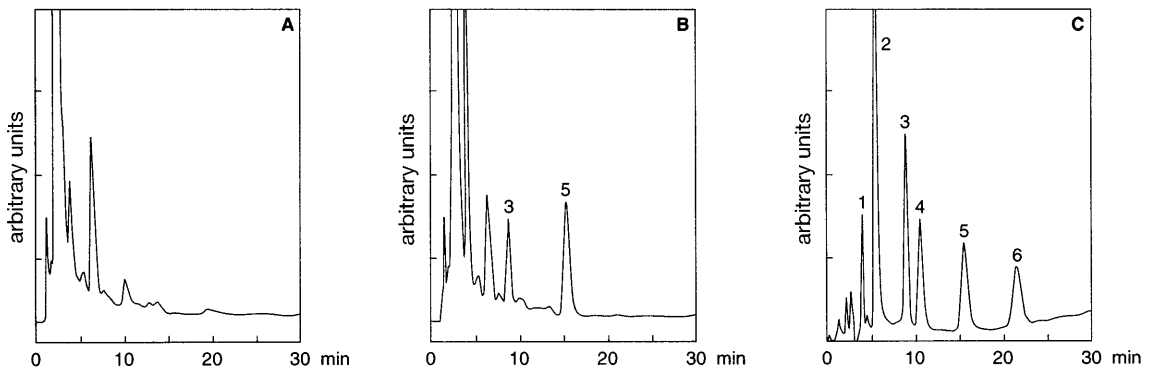


Fig. 2 A–C Chromatograms of rat urine for the in vivo release study. **A** blank sausage, **B** sausage prepared from dough containing 10 mg SDM kg–1, **C** standards of 4′-hydroxy-SDM (*1*), 5′-hydroxy-SDM (*2*), SDM (*3*), hydroxy-desamino-SDM (*4*), *N*4-acetyl-SDM (*5*), desamino-SDM (*6*)

product, SOH, could not be determined by this method due to interference with matrix components.

Nature of bound residues

It has been demonstrated that SDM is able to react with the additives glucose [17] and nitrite [18] during the preparation of raw, fermented sausage.

Products of both reactions can react with proteinaceous matter to produce bound residues of SDM. SDM primarily reacts with nitrite to give a diazonium salt which may bind to functional groups of tyrosine, histidine, cysteine and methionine moieties in proteins [8, 19]. Diazonium salts may react with compounds, such as histidine or tyrosine, in an electrophilic aromatic substitution to form azo compounds. The reaction with sulphhydryl compounds proceeds by way of a nucleophilic aromatic substitution [20]. As for the reaction with glucose, N^4-glucopyranosyl-SDM may undergo an Amadori rearrangement. The resulting compound, or its degradation products, may subsequently react with free amino groups of proteins. From these degradation and condensation products, the original amine may be released under acidic conditions.

In vitro release under acidic conditions has been shown to occur. It may be that the release of SDM from glucose is more likely to occur than its release from nitrite. As for the in vivo release of SDM, not only the residues bound through the reaction with glucose can be released, but also part of the residues bound by reaction with nitrite and subsequent reaction with proteins. When SDM has been converted into diazo compounds, reduction to SDM may take place. This reduction may be performed by the gut flora or in the liver [21]. The SDM thus formed is successively metabolised or excreted via the urine.

The difference between the in vitro release of the glucose adduct and the nitrite adduct might also explain the difference in the amount of bound residues found with labelled SDM and the bound residues released by 4 M HCl.

Balance

From the data represented in this and in previous papers [14, 18] estimates could be made regarding the fate of SDM (initial level 10 mg kg^{-1}) during sausage preparation (Table 3).

Table 3 Fate of SDM during preparation of raw, unfermented sausage (initial concentration of SDM, 10 mg kg^{-1}). *DAS*, desamino-SDM; *SOH*, hydroxy-DAS

Fate of SDM	
Unchanged	30%
Leached out by brine	20%
Converted to glucose adduct	20%
Converted to DAS	2.5%
Converted to SOH	p.m.
Covalently bound	20%
Total	93%

Conclusions

This study demonstrated that a considerable proportion of the non-extractable residues of SDM in raw, fermented sausage can be released as SDM, under both weakly and strongly acidic conditions. In these extracts, no reaction products of SDM could be observed. Calculated on the basis of the original amounts of SDM added, the percentages of SDM released were in the order of 10–15% for the lower rate of addition (1 mg kg^{-1}) and 5–10% for the higher rate of addition (10 mg kg^{-1}), while the total amount of bound residues was in the order of 20% and 15%, respectively.

It was also demonstrated that a proportion of these bound residues was released when raw, fermented sausage is fed to rats, which was subsequently excreted in the urine as SDM and N4AS. The female rats excreted amounts which were comparable to those released by 4 M HCl, while the amounts excreted by male rats were considerably lower. Results obtained with female rats demonstrated that considerable amounts of bound SDM can be released in vivo. This may be of importance when discussing the significance of bound residues as contaminants in meat products.

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