XENOBIOTICA, 2002, VOL. 32, NO. 12, 1127–1138

In vivo metabolism of diallyl disulphide in the rat: identification of two new metabolites

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Received 14 March 2002

1. Diallyl disulphide (DADS), a compound formed from the organosulphur compounds present in garlic, is known for its anticarcinogenic effects in animal models.

2. The aim was to identify and analyse the metabolites produced *in vivo* after a single oral administration of 200 mg kg^{-1} DADS to rats. The organic sulphur metabolites present in the stomach, liver, plasma and urine were measured by gas chromatography coupled with mass spectrometry over 15 days.

3. Data indicate that DADS is absorbed and transformed into allyl mercaptan, allyl methyl sulphide, allyl methyl sulphoxide (AMSO) and allyl methyl sulphone ($AMSO_2$), which are detected throughout the excretion period. Overall, the highest amounts of metabolites were measured 48–72 h after the DADS administration. $AMSO_2$ is the most abundant and persistent of these compounds. The levels of all the sulphur compounds rapidly decline within the first week after administration and disappear during the second week. Only AMSO and $AMSO_2$ are significantly excreted in urine.

4. These potential metabolites are thought to be active in the target tissues. Our data warrant further studies to check this hypothesis.

Introduction

Since early times, many beneficial properties have been ascribed to garlic for human health. Recent investigations have provided a scientific basis to these properties and have shown that garlic can interfere with different processes involved in the development of cardiovascular, neoplasic and several other diseases (Reuter 1995, Amagase *et al.* 2001). The reported biological effects have been attributed to the organosulphur compounds present in the bulb or formed after cutting or chopping the bulb.

Among these compounds, diallyl disulphide (DADS) (figure 1), which accounts for 40–60% of the essential oil of garlic, has often been reported to inhibit or reduce chemically induced carcinogenesis in rodents (Le Bon and Siess 2000, Milner 2001). DADS exerts a broad spectrum of effects on several stages involved in the process of carcinogenesis, including modulatory effects on drug-metabolizing enzymes (Yang *et al.* 2001), prevention of genotoxicity (Guyonnet

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Xenobiotica ISSN 0049–8254 print/ISSN 1366–5928 online © 2002 Taylor &
http://www.tandf.co.uk/journals
DOI: 10.1080/0049825021000017902

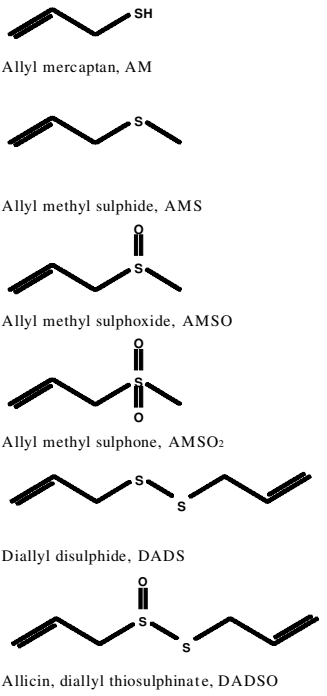

Figure 1. Structure of DADS and its metabolites.

et al. 2001), inhibition of cell proliferation, apoptosis and cell differentiation (Sundaram and Milner 1996, Lea and Ayyala 1997), and an immunostimulating effect (Kuttan 2000). In addition, DADS has also been shown to interact with cholesterol synthesis (Gebhardt and Beck 1996).

Although there is considerable knowledge about the anticarcinogenic effects of DADS, only one study has been dedicated to its *in vivo* metabolism and distribution in rodents. Pushpendran *et al.* (1980) studied the uptake and metabolic fate in mice of labelled DADS given at a sublethal dosage. They reported that DADS was rapidly absorbed, with a maximal concentration in the liver 90 min after i.p. administration. A total of 8% of the radioactivity present in the liver was identified as DADS. In addition, several *ex vivo* and *in vitro* systems have been used to analyse and identify the metabolites of DADS. In a study using an isolated perfused rat liver, DADS appeared to be converted to allylmercaptan (AM) (figure 1) (Egen-Schwind *et al.* 1992a). In primary rat hepatocytes, the metabolites of DADS have been analysed (Sheen *et al.* 1999) and within 120 min the majority of DADS disappeared from the extracellular fluid and was converted to AM and allyl methyl sulphide (AMS) (figure 1). The amount of AM was greater than the amount of AMS. Lawson and Wang (1993) reported that DADS was converted to AM in presence of blood before reaching the liver or other organs. All these results suggest that the metabolism of DADS occurred by way of reduction and methylation. Furthermore, under other experimental conditions, the oxidation of DADS has been reported (Teyssier *et al.* 1999). Previous experiments in our laboratory have demonstrated the oxidation of DADS in allicin in the presence of

human liver microsomes (Teyssier *et al.* 1999). Other naturally occurring organosulphur compounds such as diallyl sulphide (Brady *et al.* 1991) and dipropyl disulphide (Teyssier and Siess 2000) have also been shown to be oxidized. Similarly, Mitchell (1988) pointed out the importance of the *S*-oxidation of sulphides by monooxygenases as a pathway in the biotransformation of several sulphur-containing compounds.

The aim of this current study was to investigate the *in vivo* fate of DADS and its metabolites in rats after a single oral administration. For this purpose, we analysed xenobiotic distribution in various tissues and determined their pharmacokinetic parameters. Special attention was paid to the hypothetical formation of *S*-oxidized metabolites. The study was performed in rats with one oral administration of 200 mg kg^{-1} DADS. At distinct time intervals over 15 days, the concentrations of organosulphur compounds were determined in the stomach, plasma, liver and urine by gas chromatography coupled with mass spectrometry (GC-MS). The data confirmed the presence of AM and AMS as *in vivo* metabolites of DADS. Two new metabolites were detected and identified as allyl methyl sulphoxide (AMSO) and allyl methyl sulphone ($AMSO_2$) (figure 1) by different analytical approaches.

Materials and methods

Chemicals

AM, AMS, sodium metaperiodate, *p*-cymene, nonane and DADS were purchased from Sigma–Aldrich Chemical (Strasbourg, France). DADS was purified by distillation under reduced pressure (95% purity checked by GC-MS), whereas AM (80% purity) and AMS (98% purity) were used as standards without further purification. Allicin (78% purity) was synthesized by the action of metachloroperbenzoic acid on DADS as described by Mondy *et al.* (2001). All other reagents and organic solvents were of HLPC or analytical grades (SDS, Peypin, France). Dichloromethane was distilled before use for the solvent extractions.

Synthesis of AMSO and $AMSO_2$

AMSO and $AMSO_2$ were obtained by oxidation of AMS with sodium metaperiodate in a methanolic solution thawed on ice overnight (Furniss *et al.* 1989). The compounds were extracted before injection into gas chromatography–mass spectrometry (GC-MS) and gas chromatography–Fourier transformed infrared (GC-FTIR) in order to assess their structure. The reaction was total but gave a mixture of AMSO (85%) and $AMSO_2$ (15%) as estimated by GC-MS.

Animals and drug administration

Male 8-week-old Wistar rats ($n = 55$, average weight 350 g) were purchased from Janvier (Le Genest, Saint Isle, France). Rats were housed in individual stainless-wire cages, maintained at 22°C with a 12-h light/12-h dark cycle. They were fed *ad libitum* with standard diet from Harlan France (Ref. 20118S Harlan Gannat, France) and water. The animals were fasted for 18 h before and 6 h after drug administration. DADS, dissolved in corn oil, was orally administered at a dose of 200 mg kg^{-1} body weight. Negative controls were included for seven animals in order to check that no sulphur compounds were detectable in their tissues. After oral administration, animals were maintained separately in metabolic cages. Urine (kept under cold conditions) was collected every 24 h and stored at −20°C until further analysis. Animals were killed by exsanguination under anaesthesia (isoflurane 2.5% in oxygen) 10 ($n = 3$), 20 ($n = 3$), 40 min ($n = 3$), 1 ($n = 3$), 2 ($n = 3$), 6 ($n = 3$), 24 ($n = 3$), 48 ($n = 4$) and 72 h ($n = 3$) after oral dosing and then every 2 days ($n = 3$) during the 12 remaining days of the experiment. Livers and stomachs were removed, weighed and stored at −20°C until further analysis. Blood and urine were immediately centrifuged and were also stored at −20°C before analysis.

Sample preparation

Sample preparation was performed as described by Teyssier and Siess (2000). Briefly, stomachs and livers were homogenized with a high-speed blender in distilled water. A total of 100 µL from each

solution containing *p*-cymene (0.17 mg ml^{-1}), used as an internal standard (Martin-Lagos *et al.* 1995), was added to the homogenate. After adding 10% w/v trichloroacetic acid 30%, the homogenate was centrifuged and the supernatant extracted three times with dichloromethane. The same protocol without homogenization was used for the plasma samples whereas urine samples were subjected to only the dichloromethane extraction. The extracts were then concentrated by evaporation under a nitrogen stream to a final volume of 500 µl. To this volume, 100 µl nonane dissolved in dichloromethane (0.15 mg ml^{-1}) was added in order to standardize each sample concentration and an aliquot of 2 µl was then injected into the chromatograph under the conditions described below.

GC-MS conditions for DADS, AM, AMS, AMSO and $AMSO_2$ analysis

GC-MS was carried out using a Agilent Technologies (Les Ullis, France) 6890 gas chromatograph coupled to Agilent Technologies 5973 Mass Selective Detector using a capillary DB1701 column (30 m × 0.32 mm i.d., 1 µm film thickness), a splitless injection and an electronic ionization at 70 eV. The oven was programmed from 35°C (2 min) to 220°C at a rate of 5°C min^{-1}. The other conditions were: carrier gas, helium (constant velocity fixed at 35 cm s^{-1}); and temperature of the injection port, 200°C. Peak identifications were based on comparison with retention times and spectra of standards. Each sample was analysed twice. For the first acquisition, GC-MS was used in scan mode from m/z 28–300 amu in order to ascertain the identification of compounds by comparison with standards. For the second acquisition, GC-MS was used in selected ion monitoring (SIM) mode. The quantification of AM, AMS, AMSO, $AMSO_2$ and DADS was achieved by monitoring the ions at m/z 74, 88, 104, 120 and 146, respectively. The purity was continuously checked by the control of the ratio between this quantifying ion and another one, being specific for each compound. Chemical ionization was performed with NH_3 as gas reactant.

GC-MS conditions for allicin analysis

For allicin analysis, we applied the recently developed specific GC-MS method (Mondy *et al.* 2001). Briefly, analyses were carried out on a benchtop Perkin Elmer turbomass system with a split–splitless injector and a fused-silica capillary column (10 m × 0.32 mm) with 4 µm methylsilicon coating. The carrier gas was helium; the oven temperature programme was 5°C min^{-1} from 70 to 250°C. The injection port temperature was 200°C. Total ion chromatograms and mass spectra were recorded in the electron impact ionization mode of 70 eV. The transfer line and source temperature were maintained at 150°C. Peak identifications were based on comparison with retention times and spectra were obtained with pure allicin. These GC-MS conditions allowed the direct detection of allicin and the detection of 3-vinyl[4]-1,2 dithiin and 2-vinyl[4]-1,3 dithiin, two compounds formed from the allicin degradation during GC-process (Mondy *et al.* 2001). The limit of detection of allicin was estimated with a standard solution at 15 ng.

GC-FTIR conditions for AMSO and $AMSO_2$ analysis

AMSO and $AMSO_2$ from dichloromethane extracts were identified by GC-FTIR analysis using a Biorad Digilab FTS 60A spectrometer. This equipment was connected by means of a digilab Tracer® direct-deposition interface to a Hewlett-Packard HP 5890 Series II (Palo Alto, CA, USA) gas chromatograph, which was equipped with a splitless–split injector. GC separation was similar to the above-mentioned method (GC-MS conditions for DADS, AM, AMS, AMSO and $AMSO_2$ analysis).

Quantitative determination of the organosulphur compounds

For each sulphur compound, the sensitivity and linearity of the GC-MS detector used in the SIM mode was calculated from a series of standard solutions dissolved in dichloromethane. The response coefficient (a) was calculated from the curve $y = ax + b$, where y is the adjusted area (with the areas of *p*-cymene and nonane) of the quantified ion and x is the quantity of the compound (ng). The response coefficient of all other compounds was essentially similar, between 1.24 and 4.19, except for $AMSO_2$, which was low (0.32). The lowest correlation coefficient (r^2) was about 0.995. The limits of detection varied depending on the sulphur compounds and were 0.04 ng for DADS, 0.64 ng for AM, 0.04 ng for AMS, 0.03 ng for AMSO and 0.11 ng for $AMSO_2$.

The concentration of each sulphur compound in the biological samples was calculated by the ratio between the area of the sulphur compound and the product of the *p*-cymene area and the nonane area. After excluding every value beneath the linearity region of the detector, the amount of each compound was obtained by integrating its specific response coefficient. Results were given as a mean of three separate experiments (µg g^{-1} organ).

Pharmacokinetic analysis

The non-compartmental method was used to calculate the pharmacokinetic parameters with the Kinetica® software (InnaPhase, Champs sur Marne, France). The area under the plasma concentration–time curve (AUC) was computed using the trapezoidal log–linear method, when $Cn > Cn-1$. The elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2 L_z^{-1}$, where L_z is the elimination rate constant. The plasma clearance (Cl_p) was calculated as $Cl_p = \text{dose AUC}^{-1}$. T_{max} corresponded to the time for which the concentration was maximum (C_{max}).

Results

Identification of volatile metabolites

After DADS administration to rats, the parent molecule was detected in almost all analysed tissues within the first hours (data not shown). In addition, the DADS metabolites such as AM, AMS and two unknown compounds were detected in all the tested organs. Despite the specificity of the method used, neither allicin nor vinyl dithiines were detected. Figure 2 shows a gas chromatogram of the volatile components detected in the stomach. DADS, AM and AMS were identified by comparing their retention times and mass spectra with those of standard compounds. The molecular weights of the two unknown compounds, 104 and 120, were determined by chemical ionization in GC-MS. Owing to the absence of the corresponding standards, their identification was achieved by different approaches summarized in table 1. Based on GC-MS data (figure 3), the intensity of the ion at

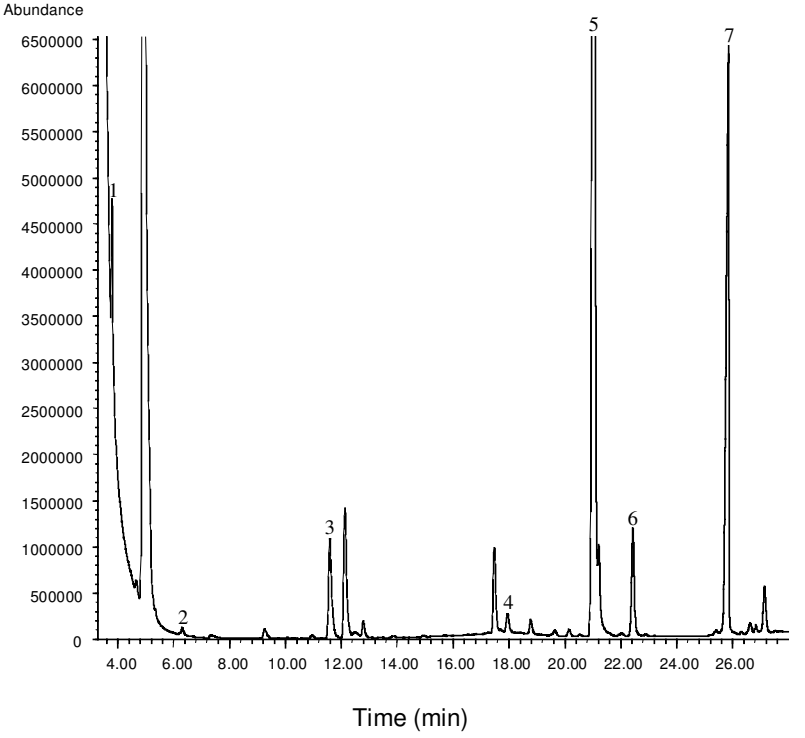

Figure 2. Total ionic current chromatogram of extracted stomach (second day) obtained on GC-MS. Peaks 1, AM; 2, AMS; 3, nonane; 4, *p*-cymene; 5, DADS; 6, AMSO; 7, $AMSO_2$. *Unidentified compounds detected both in extracts originating from control and DADS-

Table 1. Chemical characteristics of the AMSO and $AMSO_2$ metabolites.

	MW	CI[a] $(MH)^+$	Isotopic ratio (%)[b]	Formula
AMSO	104	105	106/105 5.95 (5.36) 107/105 4.71 (4.60)	C_4H_8SO
$AMSO^2$	120	121	122/121 5.80 (5.40) 123/121 4.72 (4.80)	$C_4H_8SO_2$

[a] Chemical ionization.

[b] Reported values (relative abundance) correspond to the isotopic ratio of the pseudo-molecular ion $(MH)^+$. The theoretical values are given in parentheses

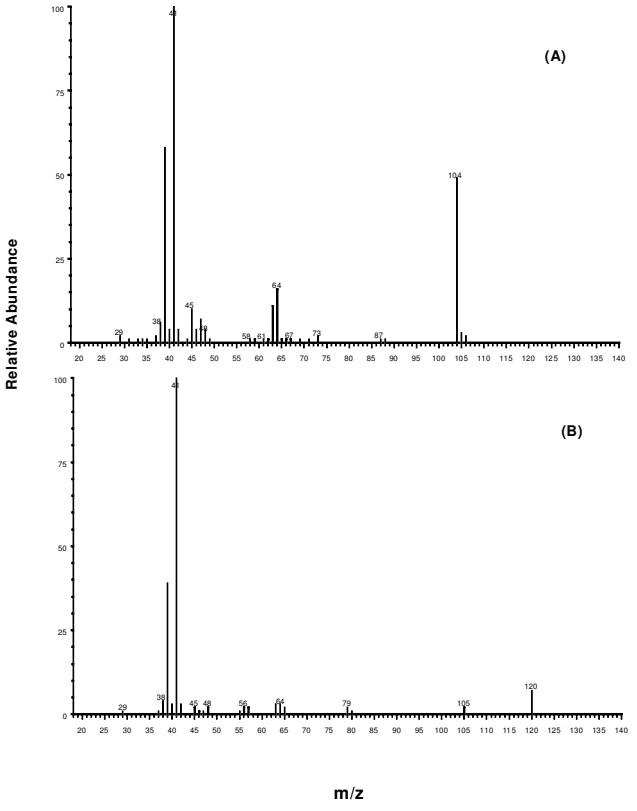

Figure 3. Mass spectra of AMSO (A) and $AMSO_2$ (B).

the m/z ratio of (M + 2) indicated the presence of one sulphur atom in each compound. The observed isotopic ratios were in agreement with the calculated ratios for molecules containing one atom of sulphur, four atoms of carbon, eight atoms of hydrogen and one or two atoms of oxygen for the first and the second metabolites, respectively. Since oxidation of sulphur compounds has been reported in the literature (Williams *et al.* 1966, Brady *et al.* 1991, Nickson *et al.* 1995, Teyssier and Siess 2000), we hypothesized that these two unknown metabolites corresponded to AMSO and $AMSO_2$. Subsequently, the chemical synthesis of these compounds was undertaken. Their chemical structures were ascertained by GC-MS and GC-FTIR. Indeed, the infrared spectra showed strong absorption bands for the sulphoxide moiety ($\nu_{S=O}$ = 998 cm^{-1}), for the sulphone moiety

(symmetric stretching ν_{SO_2} = 1136 cm^{-1}; asymmetric stretching (ν_{SO2} = 1289 cm^{-1}) and small absorption bands for the allyl chain ($\nu_{C=C}$ = 1641 cm^{-1}; $\nu_{=C-H}$ = 3088 cm^{-1}) (Socrates 1994). Hence, the *in vivo* metabolites were then compared with the synthesized standards and were identified as AMSO and $AMSO_2$.

Metabolite profiles in stomach, liver, plasma and urine

Figure 4 shows the time-dependent tissue concentrations of DADS and its metabolites AM, AMS AMSO and $AMSO_2$ after DADS oral administration. In the stomach, all these compounds were detected with various rates of appearance

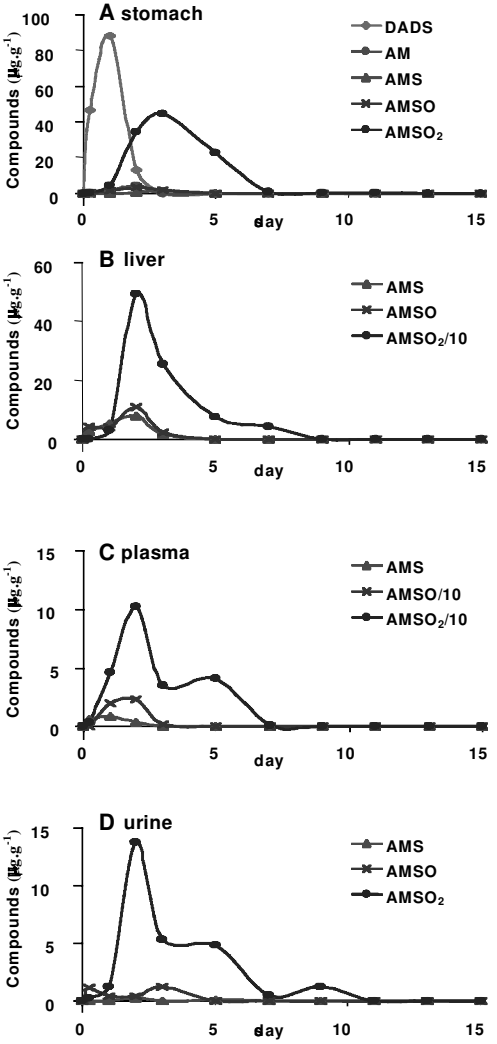

Figure 4. Concentrations of volatile metabolites in the stomach, liver, plasma and urine after one oral administration of DADS to male rats (200 mg kg^{-1}). Results are the mean of three separate experiments (μg g^{-1} organ).

and different concentration–time profiles (figure 4A). The highest levels of DADS were found during the 24 h. Thereafter, it sharply decreased and was not detectable 3 days after oral administration. In contrast, $AMSO_2$ reached a maximal concentration later in the period of follow-up, i.e. on the third day, and was still detectable for 11 days (figure 4A). In the plasma and the liver, $AMSO_2$ was the most abundant and persistent compound. The other compounds were, by rank order: AMSO and then AMS. In plasma, AM was barely detectable and DADS appeared transiently at 20 min after administration (data not shown). $AMSO_2$ concentration reached its maximum on the second day and thereafter decreased, formed a plateau between days 3 and 5 and became undetectable on day 9 (figure 4C). In liver, DADS and AM were detected only during the first hours (data not shown). The levels of $AMSO_2$ increased extensively over the first 2 days (figure 4C). After reaching a maximum, the level of $AMSO_2$ slowly decreased but was still detected 11 days after DADS administration. In urine, neither AM nor DADS were detected (figure 4D). Only trace amounts of AMS were excreted only on the second day, whereas AMSO and $AMSO_2$ were excreted throughout a longer period. The $AMSO_2$ concentration–time profile was similar to the one observed in plasma with its highest level in urine corresponding to 15% of the maximal plasma level (figure 4C, D).

Pharmacokinetic parameters

The plasma concentration–time curve of the metabolites of DADS after oral administration to the rat is shown in figure 5 and the pharmacokinetic parameters are summarized in table 2. In the case of DADS, only C_{max} and T_{max} were determined since the plasma concentrations were too low to enable an accurate determination of other pharmacokinetic parameters. The C_{max} of the metabolites were higher than that of DADS (0.001 mmol l^{-1}) as were plasma T_{max}. Indeed, the

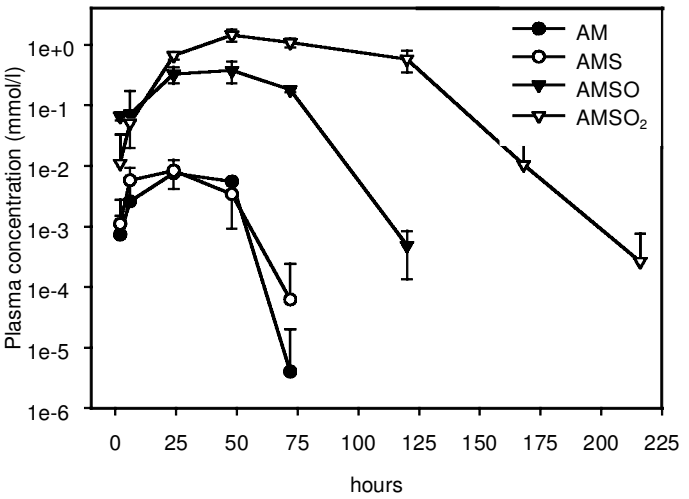

Figure 5. Plasma concentration–time curve of metabolites of DADS after one oral administration of DADS (200 mg kg^{-1}) to male rats. Data are the means of three separate experiments ± SEM. After 75 h for AM and AMS, and after 125 h for AMSO, the measured amounts were beneath the detection limit.

Table 2. Pharmacokinetic parameters of DADS and its metabolites after a single oral administration.

	DADS	AM	AMS	AMSO	$AMSO_2$
$t_{1/2}$(h)	n.d.[a]	4.39	6.78	7.16	8.64
C_{max} (mmol l^{-1})	0.001	0.008	0.008	0.376	1.440
T_{max}(h)	< 1[b]	24	24	48	48
AUC total (h mmol l^{-1})	n.d.	0.324	0.328	23.75	116.86
Cl_p($l\,h^{-1}$)	n.d.	1.475	1.455	0.020	0.004

Parameters were calculated from three separate experiments.
[a] Not determined; [b] estimated value.

DADS T_{max} was estimated to be < 1 h whereas this time increased to 24 h for AM and AMS and to 48 h for AMSO and $AMSO_2$. The AUC for AM of 0.324 h mmol l^{-1} increased for the other metabolites until the maximal value of 116.86 h mmol l^{-1} was determined for $AMSO_2$.

Discussion

The present study was carried out to investigate the *in vivo* biotransformation of DADS after oral administration to rats. For this purpose, we applied a quantitative and sensitive GC-MS method suitable for organosulphur compound analysis, which has already been validated (Martin-Lagos *et al.* 1995). We measured not only the amounts of DADS, but also those of AM, AMS, AMSO and $AMSO_2$, which were identified as DADS metabolites in the stomach, plasma, liver and urine. Based upon their plasma concentrations, their pharmacokinetic parameters were determined. We observed large interindividual variations which are particularly representative in the bioavailability (coefficient of variation of DADS, n.d.; AM, 81%; AMS, 32%; AMSO, 38%; $AMSO_2$, 7%). Our results, reported herein, show that DADS is absorbed, but owing to its low plasma concentrations, it was not possible to determine accurately the related pharmacokinetic parameters. However, the uptake of DADS in the liver was observed only during the first 2 h after dosing (data not shown), and DADS was transiently detected in plasma and was totally undetectable in the urine. The DADS levels in the liver and plasma did not exceed 0.5% of the DADS level in the stomach. These data are in accordance with a previous study in humans in which no DADS was detected in the blood and urine after ingestion of raw garlic (Amagase *et al.* 2001). The fact that DADS was hardly detected in any sample could be linked with its metabolism. Indeed, in an experiment in our laboratory using an isolated rat liver perfused with DADS, the apparent $t_{1/2}$ of DADS was estimated as < 1 h (unpublished data). In spite of the impossibility of assessing the *in vivo* pharmacokinetic parameters of DADS, all these observations taken together strongly suggest that once absorbed, DADS is rapidly and extensively metabolized.

Our data show that DADS is transformed into AM, AMS, AMSO and $AMSO_2$. Among these metabolites, $AMSO_2$ and AMSO are the major and more persistent volatile metabolites found in all assayed tissues. $AMSO_2$ appears to be the final metabolite since no new metabolite was detected when $AMSO_2$ levels decreased. In the same way, Nickson *et al.* (1995) reported that only dipropyl sulphone was detected in the urine of rats after oral ingestion of dipropyl sulphoxide, indicating the absence of subsequent biotransformation of the sul-

phone. AM and AMS were actually detected, but their bioavailabilities (table 2) were lower than those of the *S*-oxidized metabolites (table 2). The rapid elimination of AM and AMS in the breath described by Rosen *et al.* (2001) could explain their moderate amounts and low bioavailability observed in our study. However, an alternative hypothesis is that AMS is the precursor of AMSO and $AMSO_2$. This hypothesis is consistent with the T_{max} of the metabolites which are compatible with sequential DADS transformation into AM to AMS to AMSO and finally to $AMSO_2$ (table 2).

Interestingly, as far as pharmacokinetic profiles were concerned, AMSO and $AMSO_2$ presented similar patterns. In the liver and the plasma, they were characterized by an initial sharp rise during the first 2 days followed by a slow decline lasting 3–9 days suggesting a slow elimination. This phenomenon was unexpected since the overall hydrophilic nature of *S*-oxidized metabolites would favour a rapid and complete urinary excretion. However, owing to the charge separation of sulphur-oxygen linkages in the *S*-oxidized metabolites, it is possible that these metabolites may interact more strongly with proteins and lipids and thereby be retained in the body, delaying their overall excretion. Similar pharmacokinetic results have been described during a study in rats on the bioavailability of vinyldithiins, other transformation products of allicin (Egen-Schwind *et al.* 1992b). These latter compounds are additional transformation products of allicin with elimination half-lives of about 5 h and were detected in many tissues over 24 h, but the maximal serum concentration was observed within the 30 first min after ingestion. In any case, the poor elimination of the sulphone correlates with the persistence of these sulphur compounds in plasma enabling their availability to the organism.

In good agreement with the literature, our study confirms that the liver plays a major role in the *in vivo* fate of DADS not only in terms of metabolism, but also as a storage site. Indeed, the liver was the organ with the highest concentrations of all sulphur compounds (figure 4) and this phenomenon is further highlighted when the organ weight is taken into account. Moreover, the close quantitative and qualitative profiles obtained in the liver, plasma and urine for sulphur compounds strongly suggest that their output from the liver is performed by the plasma, and subsequently to the urine. The well-known poor elimination of sulphone by the kidney before the passing on to urine is also observable in our study by the persistence of these sulphur compounds in plasma.

This study has clearly established the presence of volatile metabolites in several organs after DADS ingestion but the formation of other metabolites cannot be ruled out. In particular, it has been reported that several glutathione conjugates are formed from diallyl sulphide (Jin and Baillie 1997) and dipropyl disulphide (Teyssier and Siess 2000), which could also happen during the DADS metabolic process. Hence, if those conjugates actually exist, it would be of major interest to compare their levels with those of AMSO and $AMSO_2$.

In conclusion, we confirmed that AM and AMS are *in vivo* metabolites of DADS and identified AMSO and $AMSO_2$ as new and predominant metabolites of DADS *in vivo* in rats. Previously, DADS has been assumed to be one of the active components of garlic *in vivo*. Nevertheless, owing to the rapid and extensive hepatic metabolism described herein, there seems to be no systemic bioavailability of DADS after oral administration. Taking into account the time of exposure and quantity of the different metabolites of DADS, the oxidized compounds are

probably involved in the beneficial effects ascribed to garlic. Further studies are needed to assess the biological effects of these metabolites of DADS.

Acknowledgements

The authors thank Etienne Sémon and Joëlle Chevalier for technical assistance. This research was carried out within the framework of the EU Garlic & Health project (QLK1-CT-1999-00498). The authors acknowledge the financial support of the EU.

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