METABOLISM AND DISPOSITION STUDIES WITH A [^{35}S]-LABELLED GROWTH PROMOTOR: BIS[(N^1-METHYL-N^2-METHYLSULPHONYL) GUANIDINYL ETHYL] DISULPHIDE

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SUMMARY

The disposition and metabolism of the [^{35}S]-labelled growth promotor bis[(N^1-methyl- N^2- methylsulphonyl) guanidinylethyl] disulphide was studied in the rat following oral administration. There was rapid and significant absorption of drug-derived products evidenced by maximum concentrations for plasma and the majority of sampled tissues at 1h post dose, and extensive renal clearance, with > 62% of dose voided in urine in 12h.

Analysis of urine revealed that the administered compound had been completely metabolised to five metabolites of which the two major products have been characterised. A metabolic pathway involving reductive cleavage of the disulphide bond, followed by S-methylation and sulphoxidation would appear to be involved in the biotransformation of the compound.

I. INTRODUCTION

Bis[(N^1- methyl- N^2- methylsulphonyl) guanidinylethyl] disulphide (BMD) was originally under investigation as a potential growth promotor in pigs and poultry /1,2/. Supporting disposition studies in chickens and rats, employing non-radiolabelled drug, had failed to detect the product in tissues and excreta using conventional analytical techniques /3/. The aim of the study reported here, was to evaluate the disposition and metabolism of the product in rats following single oral administration of [^{35}S] BMD (Fig. 1) at a level of 2 mg/kg.

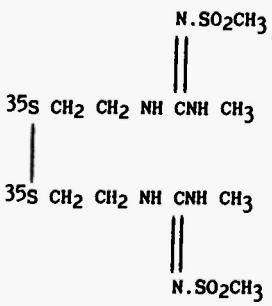

Fig. 1: [^{35}] BMD.

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II. MATERIALS AND METHODS

2.1 Radiolabelled Materials

[^{35}S] BMD was synthesised from [^{35}S] cystamine (supplied by Amersham International, UK) by the Radiochemistry Department, Rhône-Poulenc Ltd., U.K. The resulting product had an initial specific activity of 26.0mCi/mmole and a radiochemical purity of 98%. In addition, no chemical impurities could be detected by chromatographic means.

2.2 Animals

Male albino Sprague-Dawley CD rats (weight ca. 225g) were obtained from Charles River U.K. Ltd. Spratts Laboratory CRX diet were supplied *ad libitum* to animals prior to and during the study. Animal experimentation was conducted within Home Office guidelines.

2.3 Study Design

[^{35}S] BMD formulated as a 1 mg/cm^2 suspension in tragacanth mucilage was administered at a level of 2mg/kg p.o. to five groups of 3 rats. Each group was placed in a Jencon's metabowl (with free access to food and water), permitting the separate collection of urine and faeces (cooled with dry ice). In addition, for the group to be sacrificed at the latest time post dose, an attempt was made to trap volatile acidic ^{35}S oxidation products by drawing expired air through Nilox columns containing aqueous 5M ethanolamine. Subsequently, one group was sacrificed at 1, 4, 12, 24 and 48h post dose, in order to follow the time course of the product's absorption, distribution and excretion. Excreta voided by each group up to the time of sacrifice were determined for their radioactivity contents by means of liquid scintillation counting. Individual tissues from the 3 animals constituting a sacrifice group were pooled, processed and then determined for radioactivity content. Urine was subsequently analysed chromatographically and the metabolite profile established.

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2.4 General Chemicals and Equipment

The scintillation cocktail employed in the measurement of radioactivity was Instagel (Packard Instrument Co. U.S.A.). General chemicals and solvents were supplied by Rhône-Poulenc Ltd., U.K. Tissue and faecal samples where appropriate were homogenised and replicate aliquots then solubilised and decolourized with an hydrogen peroxide/perchloric acid mixture (0.4 and 0.2ml), prior to liquid scintillation counting on a Beckman LS 7800 system (Beckman Instruments Inc., U.S.A.). These samples were counted against a quench curve prepared by spiking Instagel with identical amounts of [^{35}S] standard but increasing amounts of the quenching agent, CCl_4. There was no measurable loss of radioactivity on similarly processed controls spiked with known levels of [^{35}S] BMD.

Qualitative tlc was performed on Polygram SIL G and SIL G UV_{254} plates supplied by Macherey-Nagel & Co., W. Germany. Preparative tlc was performed on prewashed precoated silica gel G precoated plates obtained from Anachem Ltd., U.K.

2.5 Chromatography

Urine and corresponding extracts which were applied to precoated silica tlc plates were analysed in the following solvent systems:

A. H_2O : NH_3 (33% w/w) : propan-2-ol	(2:1:20, v/v)
B. ethylacetate : acetic acid : butan-1-ol : H_2O	(1:1:1:1, v/v)
C. H_2O : NH_3 (33% w/w) : propan-2-ol	(2:2:30, v/v)
D. H_2O : NH_3 (33% w/w) ethanol : butan-1-ol	(10:1:21:35, v/v)
E. propan-2-ol : NH_3 (33% w/w) dichloroethane	(45:1.5:45, v/v)

Unlabelled BMD, its reduction product 2-[(N^1- methyl- N-^{2} methylsulphonyl) guanidyl] ethane- 1-thiol (MSG, Fig. 2) and [^{35}S] cystamine were available as authentic reference standards for chromatography. Both BMD and MSG were synthesised by Pharmaceutical Chemistry, Rhône-Poulenc Ltd. Radioactive products on developed tlc plates were localised by autoradiography using Ultra-film [^{3}H] photographic film (LBK Instruments, U.K.). Unlabelled putative metabolites were detected with Ninhydrin spray reagent (0.2% in ethanol, Aldrich Chemical Co. Ltd.) and/or visuallsed under UV_{254} light.

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2.6 Isolation of Metabolites

Post administration urine (the 0-24h collection from the group sacrificed at 48h) was applied to pre-washed C_{18} SEP-PAK cartridges and subsequently washed with methanol. Average recovery of applied radioactivity was determined to be 100.5%; with 19.9% associated with the aqueous filtrate and 80.6% present in the methanol phase. The methanolic extract was applied to tlc plates which were subsequently developed in solvent system C. Once developed, bands corresponding to the two major metabolites (localised by autoradiography) were scraped off the plates and the silica extracted with methanol. Each partially purified band was re-applied to fresh preparative plates and the procedure repeated twice more, once with solvent C and then with solvent E, in order to obtain purified metabolites.

2.7 Physico-Chemical Analyses

Authentic BMD and purified metabolites were subjected to: ^{1}H nuclear magnetic resonance spectroscopy on a Varian XL-200 spectrometer, infra red spectroscopy on a Pye-Unicam SP4000 spectrophotometer and mass spectrometry on a VG-Micromass 12B spectrometer using direct probe insertion, at an ionisation potential of 70eV and a source temperature of $200^{0}C$.

III. RESULTS

Following oral administration of [^{35}S] BMD, drug-derived radioactivity was rapidly absorbed from the gastro-intestinal (g.i.) tract into the systemic circulation. A plasma C_{max} of 0.64 μg/ml was observed at 1h post dose (Table 1).

The level of urinary excretion of radioactivity suggested considerable absorption of drug-derived products (> 70% of dose) followed by rapid renal clearance (Table 2).

There was rapid distribution of absorbed drug-derived products into all tissues sampled, with maximum concentrations generally observed 1 h post dose (Table 3). The majority of body content of radioactivity was associated with the g.i. tract and contents, at all sacrifice times.

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TABLE 1

Mean Concentrations of Radioactivity in Plasma and Blood Following Oral Administration of [^{35}S] BMD

Time of Sacrifice (h)	µg equivalents/ml	
	Plasma	Blood
1	0.639	0.507
4	0.285	0.234
12	0.064	0.058
24	0.011	0.013
48	0.004	0.007

Data presented result from the analysis of pooled samples from the 3 animals constituting each sacrifice group.

TABLE 2

Distribution and Excretion of Radioactivity Following Oral Administration of [^{35}S] BMD

	% of dose recovered at				
	1h	4h	12h	24h	48h
Total body content	79.79	45.26	20.74	5.6	3.35
Faeces	0.02	0.24	0.57	7.51	7.91
Urine	4.38	32.15	62.33	70.85	74.98
Expired Air	n.c.	n.c.	n.c.	n.c.	0.13
Cage washes	3.48	10.15	4.18	1.26	0.56
Total	84.67	87.70	87.80	85.20	86.93

n.c. = not collected.

Data presented refer to the analysis of collective excreta voided by the 3 animals of each sacrifice group.

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TABLE 3

Distribution of Radioactivity (expressed as ug eqivalents/g) Following Oral Administration of [^{35}S] BMD

Tissue	ug equivalents /g tissue at 1h	4h	12h	24h	48h
Blood	0.5065	0.2340	0.0583	0.0125	0.0074
Adrenal	0.7191	0.3476	0.1380	0.0719	0.0963
Bladder	2.7951	1.3362	0.5801	0.1183	0.0286
Eye	0.1962	0.1892	0.0711	0.0247	0.0171
Pituitary	1.4954	0.5702	0.4604	0.4518	0 7133
Thyroid	0.7868	0.6992	1.2604	0.4514	0.5023
Adipose	0.1376	0.1616	0.0656	0.0140	0.0164
Brain	0.0948	0.0839	0.0568	0.0143	0.0120
Carcass	0.2938	0.3072	0.0879	0.0513	0.0239
Heart	0.3835	0.2813	0.0769	0.0227	0.0237
Kidney	1.0636	0.5778	0.2334	0.0371	0.0293
L.int	0.6504	3.9957	3.4203	0.6100	0.2312
L.i.con	0.1956	10.9191	12.2994	2 8624	1.8232
Liver	0.3264	0.3666	0.1103	0.0231	0 0205
Lung	0.7372	1.3383	0.0950	0.0314	0.0186
Muscle	0.3290	0.1870	0.0690	0.0242	0 0235
Pancreas	0.4808	0.3269	0.1030	0.0474	0 0171
Sm.int	9.5864	2.7926	0.2411	0.1757	0.1017
S.i.con	14.5125	8.6974	0.4026	0.7798	0.4470
Spleen	0.4187	0.3693	0.1394	0.0434	0.0242
Sto.con	32.2628	8.5116	0.1405	3.7340	0.4400
Thymus	0.5205	0.3260	0.1146	0.0326	0.0187

Abreviations :
S.i.con = Small intestinal contents
L.i.con = Large intestinal contents
Sto.con = Stomach contents
Muscle = Skeletal muscle

Data presented, results from the analysis of pooled samples from the 3 animals constituting each sacrifice group.

Low levels of radioactivity persisted in all tissues up to 48h post dose (3.35% of dose), although the majority by this time had been eliminated predominantly in urine 75% and to a lesser extent in faeces 7.91%.

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Chromatographic analysis of urine followed by autoradiography revealed the complete absence of unchanged parent compound, its reduction product MSG and cystamine, although two major metabolites U2 (constituting 66.6% of urinary radioactivity) and U5 (23.4%) plus 2-3 minor metabolites (collectively 10%) were detected (Table 4).

TABLE 4

Rf Values of Urinary Metabolites and Authentic Standards

	Rf values in solvents			
Metabolite	A	B	C	D
U1	0.46	n.d.	0.43	n.d.
U2	0.58	0.51	0.51	0.34
U3	n.d.	n.d.	n.d.	0.44
U4	n.d.	n.d.	n.d	0.48
U5	0.80	0.80	0.78	0.75
Standards	A	B	C	D
Cystamine	0.41	0.32	0.29	0.00
BMD	0.76	0.74	0.73	0.64
MSG	0.67	0.75	0 64	0.58

n.d. = not detected

Note: Each value is the mean of two determinations.
Composition of solvents is given in Section 2.5.

Proton nuclear magnetic resonance spectroscopy of isolated metabolites U2 and U5 indicated a number of signals which were assigned as indicated in Tables 5 and 6.

Mass spectral analysis of metabolites U2 and U5 revealed peaks at the following m/z values:

Metabolite U2 : 242 (MH^+) ; 226 ($M, -CH_3)^+$; 224 ($M,-OH)^+$; 208 ($M,-OH, -O)+$; 178 ($M,-CH_3SO)^+$; 177 ($M,-CH_3SOH$) ; 162, 152, 135 and 121

Metabolite U5 : 226 (MH^+) ; 225 (M^+) ; 210 ($M.-CH_3)^+$; 178 ($M,-SCH_3)^+$; 164 ($M,-CH_2SCH_3)^+$; 152 ($M,-CH_2 = CH.S.CH_3)^+$ and 135 ($152-NH^3$)

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TABLE 5

^{1}H Nuclear Magnetic Resonance Analysis of Metabolite U2

Metabolite U2:

Chemical Shift	Multiplicity	Integration	Assignment
7.10	Broad Signal	1H	– N*H* –
6.38	Broad Signal	1H	– N*H* –
3.88	Multiplet	2H	– $SOCH_2CH_2$ NH –
3.10	Multiplet	1H	– SO CH_2CH_2NH – (one CH of non-equivalent pair)
2.94	Singlet	3H	CH_3SO_2N=C
2.80	Doublet, J=4Hz	3H	CH_3–NH
2.76	Multiplet	1H	SO CH_2CH_2NH (one CH of non-equivalent pair)
2.66	Singlet	3H	CH_3–SO

TABLE 6

^{1}H Nuclear Magnetic Resonance Analysis of Metabolite U5

Metabolite U5:

Chemical Shift	Multiplicity	Integration	Assignment
6.90	Broad Signal	1H	– N*H* –
5.30	Broad Signal	1H	– N*H* –
3.50	Quartet, J=6Hz	2H	–S–CH_2CH_2–NH
2.96	Singlet	3H	CH_3SO_2N=C
2.84	Doublet, J=4Hz	3H	CH_3NH
2.70	Triplet, J=6Hz	2H	–S–CH_2CH_2–NH
2.12	Singlet	3H	CH_3–S

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Infra red spectra of metabolites U2 and U5 proved to be very similar and both showed the expected C—H ; N—H ; C—N and SO_3 bands. The most significant difference between the two spectra was the presence of a band at 9.9μ in the spectrum of metabolite U2 which was not seen in the corresponding U5 spectrum. This band at 9.9μ was assigned to a >S = 0 stretching absorption.

The two major metabolites U2 and U5 were therefore identified by physico-chemical means to be respectively N^1- methyl- N^3- [2-methyl-sulphinyl) ethyl]- N^2-methylsulphonyl guanidine (MMS) and N^1-methyl- N^2- methylsulphonyl- N^3- methylthioethyl guanidine (MMT). Authentic standards of these two compounds were synthesised, and used as chromatographic reference compounds to confirm the structure of the metabolites (Table 7). No attempt was made to identify the minor metabolites.

TABLE 7

Rf Values of Purified Metabolites U2, U5 and Authentic Standards

Metabolite	Rf values in solvents A	B	C	D
U2	0.41	0.49	0.38	0.33
U5	0.71	0.81	0.78	0 71
Standards	A	B	C	D
MMS	0.42	0.48	0.38	0.33
MMT	0.71	0.81	0.69	0.71

Note: Each value is the mean of two determinations.

IV. DISCUSSION

Following oral administration of [^{35}S] BMD to male rats at a level of 2mg/kg, drug-derived products are rapidly and extensively absorbed, probably by an active transport mechanism that mediates the uptake of structurally analogous amino acids.

Thereafter, drug-derived products distribute rapidly into all organs

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sampled, with low levels persisting to 48h post dose. This may be indicative of retention or alternatively of the incorporation of ^{35}S into tissues via the metabolic pool.

Nonetheless, the majority of the dose is rapidly eliminated predominantly in the first 12h, notably by renal clearance. The compound would appear to be extensively metabolised to major products which indicate a metabolic pathway involving reductive cleavage, followed by S-methylation and sulphoxidation (Fig. 2), the latter two biotransformations being well documented for other alphatic thiols /4,5/.

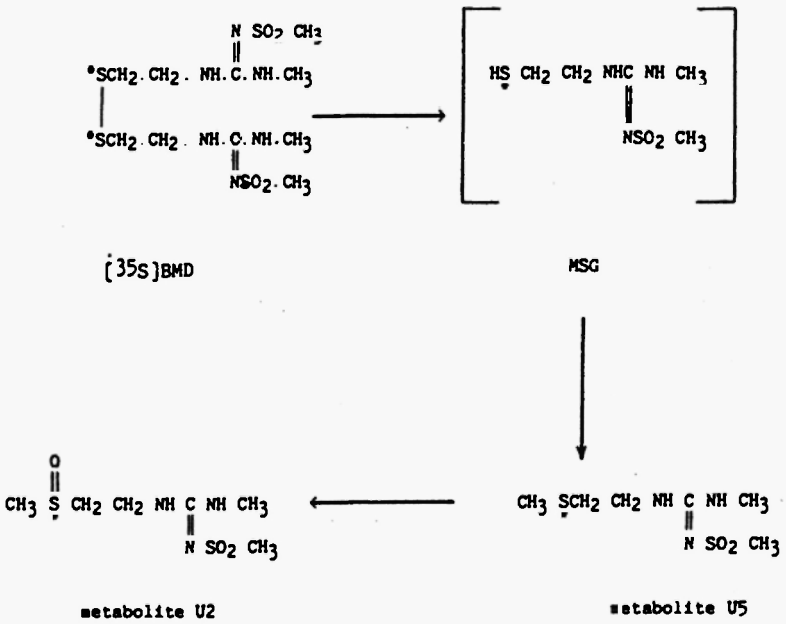

Fig. 2: Postulated Metabolism of [^{35}S] BMD.

* Indicates position of radiolabel.

Overall recoveries of administered radioactivity in tissues, excreta and apparatus washings, were in the range 85-88% of dose. These results were not considered readily acceptable in view of the normal recoveries for such ADME studies being 100 ± 5% of administered dose. It was

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considered that the production and incomplete trapping of volatile ^{35}S-labelled metabolites such as $H_2{}^{35}S$ in expired air, in systems designed to collect expired $^{35}SO_2$ and/or $^{35}SO_3$ might account for the shortfalls seen in this study. Similarly, the loss of volatile drug-derived products on sample processing remains a possibility, despite no loss of radioactivity on processing controls spiked with [^{35}S] BMD itself.

V. CONCLUSION

Although following oral administration of [^{35}S] BMD there is good absorption of drug-derived radioactivity, pre- or post-systemic metabolism of the product would appear to be extensive, probably accounting for the non-detection of parent compound in biological fluids by cold analytical techniques.

VI. REFERENCES

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