1 **Absolute bioavailability and dose-dependent pharmacokinetic behaviour of**
2 **dietary doses of the chemopreventive isothiocyanate sulforaphane in rat**
3

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Abstract

Sulforaphane is a naturally-occurring isothiocyanate with promising chemopreventive activity. An analytical method, utilising LC-MS/MS, that allows the determination of sulforaphane in small volumes of rat plasma following exposure to low dietary doses was developed and validated, and employed to determine its absolute bioavailability and pharmacokinetic characteristics. Rats were treated with either a single intravenous dose of sulforaphane (2.8 μmol/kg) or single oral doses of 2.8, 5.6 and 28 μmol/kg. Sulforaphane plasma concentrations were determined in blood samples withdrawn from the rat tail at regular time intervals. Following intravenous administration, the plasma profile of sulforaphane was best described by a two-compartment pharmacokinetic model, with a prolonged terminal phase. Sulforaphane was very well and rapidly absorbed, and displayed an absolute bioavailabilty of 82% which, however, decreased at the higher doses, indicating a dose-dependent pharmacokinetic behaviour; similarly, C_{max} values did not rise proportionately to the dose. At the highest dose used, the rate of absorption constant k_{ab}, biological half-life $t_{1/2}$ and apparent volume of distribution decreased significantly. It is concluded that in the rat orally administered sulforaphane is rapidly absorbed, achieving high absolute bioavailability at low dietary doses, but dose-dependent pharmacokinetics was evident, with bioavailability decreasing with increasing dose.

Introduction

Isothiocyanates are promising chemopreventive agents that are present in substantial concentrations in brassica cruciferous vegetables; in epidemiological studies intake of these vegetables was found to afford protection against a number of cancers[1-3]. Isothiocyanates occur as glucosinolates but are released following exposure to the enzyme myrosinase (β-thioglucoside glucohydrolase) which comes into contact with these compounds during the harvesting, chopping and mastication of these vegetables. Moreover, microbial myrosinase activity in the human intestine contributes to the release of isothiocyanates from their glucosinolate precursors[4].

Epidemiological studies revealed the potential of isothiocyanates to protect against tumourigenesis at a number of sites[5,6]. Furthermore, studies conducted in animal models established that isothiocyanates can antagonise the carcinogenicity of chemicals, including dietary carcinogens such as polycyclic aromatic hydrocarbons[7]. A number of mechanisms appear to contribute to their anticarcinogenic activity including impairment of the bioactivation of carcinogens and increased detoxication of their reactive intermediates, suppressed cellular proliferation and increased apoptosis[3,8-10]. As most of these mechanistic studies have been conducted in *in vitro* systems, utilising a range of concentrations, in order to extrapolate such data to the *in vivo* situation it is essential that the pharmacokinetic fate of isothiocyanates, following dietary levels of intake, including their bioavailability and plasma concentrations are ascertained.

One such isothiocyanate is sulforaphane [1-isothiocyanato-4-(methylsulphinyl) butane], found particularly in broccoli where it is present as the glucosinolate, glucoraphanin. In animal studies it efficiently antagonised the carcinogenicity of polycyclic aromatic hydrocarbons[11] and reduced the number of azoxymethane-induced aberrant crypt foci in rat colon[12]. Although its mechanism of action appears to be multifactorial, its chemopreventive effect also involves impairment of the initiation stage of carcinogenesis. Sulforaphane inhibited the DNA binding of benzo[a]pyrene and 1,6-dinitropyrene in human mammary epithelial cells[13] and the formation of DNA adducts with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in human hepatocytes[14]. Even when administered at dietary doses, sulforaphane stimulates detoxication enzymes such as quinone reductase[15], and at higher doses it also enhances the activity of other deactivating enzyme systems such as glutathione S-transferase[11,16]. However,

1 sulforaphane also acts at the post-initiation stages, for example suppressing the
2 conversion of lung benign tumours to carcinomas in mice[17]. An interesting
3 observation is that mercapturates, the principal metabolites of isothiocyanates, may
4 retain the anticarcinogenic activity of the parent compounds[17,18].
5 In the present study an LC-MS/MS method has been developed to determine
6 sulforaphane in rat plasma, and was used to determine the absolute bioavailability of
7 dietary doses of sulforaphane following oral and intravenous administration.
8
9 **Experimental methods**
10
11 ***Materials***
12 R,S-Sulforaphane was purchased from LKT Laboratories (St Paul, Minnesota, USA).
13 All solvents were of HPLC grade (Fisher scientific, UK).
14
15 ***Treatment of animals***
16 Male Wistar albino rats (200g) were obtained from B&K Universal Ltd (Hull, East
17 Yorkshire, UK). The animals were housed at 22 ± 2 °C, 30-40 % relative humidity in
18 an alternating 12-hr light:dark cycle with light onset at 07.00 hr. The animals were
19 allowed to acclimatise for at least 4 days before commencement of the study; they
20 were then treated, 4 per group, with either a single intravenous dose of sulforaphane
21 (0.5 mg/kg), in a volume of 100 μl of water, or single oral doses of 0.5, 1.0 and 5.0
22 mg/kg, corresponding to 2.8, 5.6 and 28 μmol/kg, dissolved in 1 ml of water. Blood
23 samples (100 μl) were withdrawn from the rat tail at regular time intervals for 8 hours,
24 and placed into lithium-heparinised centrifuge tubes. A sample was also obtained 24
25 hours after administration as well as from untreated rats.
26
27 ***Sample preparation***
28 Aliquots of the plasma (40 μl) were made to 1 ml with 0.01 M phosphate-buffered
29 saline (pH 7.3) and were subsequently extracted with chloroform (5 ml) twice for 30
30 minutes; the layers were separated by centrifugation at 510g for 10 minutes. The
31 combined chloroform extracts were evaporated under nitrogen using an N-EVAP®
32 model 111 (Organomation Assoc. Inc, USA) and redissolved in 40 μl of the HPLC
33 mobile phase (10 % acetonitrile in water containing 0.1% formic acid), and 20 μl was

1 injected for analysis. Quality control sulforaphane solutions of known concentrations
2 (0.05, 0.5 and 1.8 μg/ml) were carried through the same procedure.
3
4 ***Determination of sulforaphane in rat plasma***
5 Plasma concentrations of sulforaphane in the rat plasma were determined by LC-
6 MS/MS, using methodology developed in our laboratories. Separation of sulforaphane
7 was achieved using an Ultimate 3000 (Dionex, Camberley, UK) HPLC employing a
8 Synergi 4u Fusion-RP analytical column (80Å particle size, 150 x 1 mm) fitted with a
9 KrudKatcher in-line filter, both supplied by Phenomenex (Macclesfield, UK). Mobile
10 phase consisted of solvent A (10 % acetonitrile in water containing 0.1% formic acid)
11 and solvent B (90 % acetonitrile in water containing 0.1% formic acid). The analyte
12 was eluted with a linear gradient (0 to 20%) of organic solvent B over 22 min. The
13 column was then washed with 80% of solvent B over 3 min and re-equilibrated for a
14 further 10 min; the eluent flow rate was 0.05 ml/min. Under these conditions, the
15 retention time of sulforaphane was about 18 minutes in a total run time 35 minutes.
16 Sulforaphane was detected on-line using a API 2000 triple quadrupole mass
17 spectrometer (Applied Biosystems, Warrington, UK) equipped with a turbo-ion
18 electrospray probe, operating in the positive ionisation mode. The analyte was
19 detected by monitoring the m/z 178→ 114 and 178→72 mass transition (Figure 1) in
20 the MRM scan mode, under the following conditions: turbo ion-spray interface
21 temperature, 350°C; collision-activated dissociation gas pressure and curtain gas
22 pressure, 3 and 40 p.s.i. respectively; turbo ion-spray voltage, 5000 V; declustering,
23 entrance and focusing potential, 11, 10 and 300 V respectively; collision energy 18
24 and 41 V for m/z 114 and 72 transitions respectively; collision cell exit potential, 15
25 V. Acquisition of data was achieved using the Analyst software (version 1.4; Applied
26 Biosystems).
27
28 ***Pharmacokinetic analysis***
29 Pharmacokinetic analysis was carried out using PK solutions™ software package
30 (version 2.0, Summit Research Services, Ohio, USA). Because of the very long
31 terminal phase ($t_{1/2}$ = 65.6 hours after intravenous administration), pharmacokinetic
32 parameters were calculated from the better defined AUC_{0-24} , rather than from $AUC_{0-\infty}$,
33 to avoid introducing an inaccuracy in extrapolating the terminal phase to time infinity.

1 AUC_{0-24} was calculated using the trapezoidal rule utilising the time data determined
2 experimentally. Absolute bioavailability (F) was determined from the ratio of the oral
3 to intravenous dose-normalised AUC_{0-24} values. Apparent volume of distribution (Vd)
4 was calculated from the equation $Vd = FD/ AUC_{0-24}\ k_{el}$, where k_{el} is the elimination
5 rate constant. Plasma clearance (Cl) was determined using the equation $Cl = FD/ AUC_{0-24}$
6 . Finally, C_{max} and T_{max} were determined graphically from the plasma
7 concentration versus time plots.

8 Animal data were analysed individually, and are presented as mean ± SD;
9 statistical evaluation was performed using the Student's t-test, where n=4.

10

11 **Results**

12 A LC-MS/MS method for the measurement of sulforaphane in rat plasma has been
13 developed and validated. No sulforaphane was detected in rat plasma prior to
14 administration of the isothiocyanate. Limit of detection, defined as the lowest
15 concentration of analyte that generates a minimum signal to noise ratio of 3, and limit
16 of quantification, defined as the lowest concentration of analyte that gives rise to an
17 instrument response with a minimum signal to noise ratio of 5, were 5 and 15 ng/ml
18 respectively, following a 20 μl injection (Figures 2A and 2B). The calibration curve,
19 at a plasma concentration range of 0.01 to 2 μg/ml showed excellent linear
20 relationship, with an R value of 0.99. Recovery of sulforaphane at three plasma
21 concentrations, 0.05, 0.5 and 1.8 μg/ml, was 91, 91 and 88% respectively (n=6). At
22 the same concentrations inter-assay variation was 1.0, 2.2 and 3.2% respectively,
23 whereas intra-assay variation, established at the same concentrations, was 1.5, 6.5 and
24 5% respectively (n=4). Finally, accuracy was established by comparing calculated and
25 theoretical values at two plasma concentrations (0.5 and 1.8 μg/ml); calculated and
26 theoretical values did not differ more than 11 % (n=4).

27 Figure 3 shows the time-course changes in the plasma concentrations of
28 sulforaphane, plotted using a semi logarithmic plot, following intravenous
29 administration to rats; the plasma profile is best described by a two-compartment
30 pharmacokinetic model. Within two hours, plasma concentrations decline to about
31 10% of the levels present 0.5 hour after administration; the concentrations then remain
32 fairly constant indicating a long terminal phase. Similarly, following oral
33 administration of the same dose, a marked and rapid decline in plasma concentrations

of sulforaphane is evident between 1 hour after administration, when peak concentrations are achieved, and 2 hours after administration, followed by a prolonged terminal phase (Figure 3). At the higher doses, however, the decline in plasma concentrations, after maxima have been attained, is more gradual.

The pharmacokinetic parameters of sulforaphane following oral and intravenous administration are shown in table 1. The C_{max} and AUC_{0-24} values in orally-treated rats increased with dose, but not proportionately; rise in AUC_{0-24} and C_{max} values was lower than would be anticipated. Comparison of AUT_{0-24} values between the intravenously- and orally-treated groups, at the 0.5 mg/kg dose, indicate an absolute bioavailabilty of 82% which, however, decreased at the higher doses. Finally, the rate of absorption constant k_{ab}, biological half-life $t_{1/2}$ and apparent volume of distribution decreased at the highest dose used.

Discussion

An analytical method, utilising LC-MS/MS, that allows the determination of sulforaphane in small volumes of rat plasma following exposure to low dietary doses was developed and validated, and employed to determine its absolute bioavailability and pharmacokinetic characteristics in the rat following intravenous and oral administration. An 150g serving of fresh broccoli will release 56-112 mg of sulforaphane[19], so that the intake for a 70 kg individual would be 0.8-1.6 mg/kg. The doses employed in this study of 0.5-5.0 mg/kg were chosen to represent such concentrations of intake.

In the current studies it was considered prudent not to deprive the animals of food prior to administration of sulforaphane, as it could have an impact on its metabolic clearance. The principal pathway of metabolism of this compound involves conjugation with glutathione, followed by further processing of the conjugate to the mercapturate[20]. Withdrawal of food can result in a decline in the cellular concentrations of glutathione[21].

Following oral administration, sulforaphane peak plasma concentrations were attained at about one hour, indicating rapid absorption, compatible with its lipophilicity and small molecular size. Similarly, in studies conducted in human volunteers, isothiocyanates, measured as total dithiocarbamates, were rapidly absorbed reaching peak plasma concentrationss one hour after ingestion of broccoli sprout preparations[22]. In a recent study, where sulforaphane levels were determined in

1 volunteers consuming a broccoli soup, maxima were attained one hour after intake, in
2 concordance with the present findings in rats[23]. At the highest dose only, the
3 absorption rate constant decreased, and this may explain in part the fact that $C_{\max}$
4 values did not rise proportionately to the dose. This observation raises the possibility
5 that sulforaphane may to some extent be absorbed by a carrier-mediated transport
6 mechanism that is saturated at this dose level.

7 Oral absolute bioavailability of sulforaphane in rats was over 80% at the
8 lowest oral dose studied. It is likely that sulforaphane is subjected to modest first-pass
9 metabolism as glutathione and mercapturate metabolites of this isothiocyanate can be
10 generated by intestinal as well as hepatic enzymes, or even possibly in the blood as it
11 contains low concentrations of glutathione that can interact chemically with the
12 isothiocyanate. Studies using human jejunum *in situ* have established that
13 sulforaphane is well absorbed by enterocytes where it is conjugated with glutathione
14 during absorption and secreted back into the lumen[24].

15 In the present study, the oral bioavailability of sulforaphane was dose-
16 dependent, being only about 20% at the highest dose studied of 28 μmol/kg, i.e. a
17 quarter of that observed at a dose of 2.8 μmol/kg. These observations imply that
18 intake of sulforaphane supplements may not be as effective as envisaged in achieving
19 high plasma concentrations of the compound. Isothiocyanates display high protein
20 binding[25,26], presumably because of their facile interaction with –SH groups, and it is
21 conceivable that at the higher doses protein-binding sites are saturated so that
22 sulforaphane remains free and available for metabolism and excretion. It is relevant to
23 point out that albumin contains only a single residue of free cysteine (Cys34). The
24 observed dose-dependent decrease in biological half-life values concord with such a
25 mechanism of action. Non-linear pharmacokinetics in rats have also been reported for
26 phenethyl isothiocyanate, having an aromatic substituent[25], and collectively these
27 observations indicate that the isothiocyanate group is more likely to be responsible for
28 this effect rather than the substituent.

29 Following intravenous and oral dosing a rapid marked drop is observed in the
30 plasma concentrations of sulforaphane, and this most likely reflects cellular uptake.
31 Isothiocyanates such as sulforaphane, attain very high intracellular concentrations as a
32 result of their interaction with glutathione[16,27,28]. As the absorbed sulforaphane is
33 readily conjugated with glutathione and possibly other thiols, the concentration
34 gradient drives the further cellular uptake of the isothiocyanate, which can achieve

mM concentrations, and is accompanied by a marked drop in glutathione levels[17]. The glutathione and cysteinylglycine conjugates are exported through membrane transporters such as P-glycoprotein[29,30]. It has been demonstrated in *in vitro* studies that peak intracellular concentrations of isothiocyanates are attained within 3 hours of exposure, and intracellular concentration may be as much as 200-fold higher than extracellular concentration[3]. Such extensive intracellular localisation would explain the large apparent volume of distribution. Elimination of sulforaphane was characterised by a long terminal phase; in fact, no major difference was evident in plasma concentrations between 6 and 24 hours following intravenous administration or oral administration at the lower doses. Most likely this is a consequence of protein binding, rendering the isothiocyanate unavailable for elimination through metabolism and excretion.

In summary, the present paper demonstrates that in the rat, following oral administration of dietary doses, sulforaphane is rapidly absorbed, achieving high absolute bioavailability at low doses. However, dose-dependent pharmacokinetics was evident, with bioavailability decreasing with increasing dosage.

Acknowledgement

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30 mediated export as dithiocarbamates. *Cancer Lett.* **204**, 23-31.
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8 **Table 1: Absolute bioavailability and pharmacokinetic parameters of**
9 **sulforaphane following administration of dietary doses**
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11 Results are presented as Mean ± SD for four animals.
12 *P<0.05; **P<0.01; ***P<0.001, when compared to the 0.5 mg/kg oral dose
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14

Parameter	Intravenous (0.5 mg/kg)	Oral (0.5 mg/kg)	Oral (1.0 mg/kg)	Oral (5.0 mg/kg)
C_{max} (ng/ml)	-	46.4 ± 19.4	84.0 ± 19.6*	211.3 ± 24.3***
T_{max} (h)	-	0.5 ± 0.0	0.8 ± 0.3	1.0 ± 0.7
k_{ab} (h^{-1})	-	5.78 ± 1.70	3.74 ± 0.44	2.57 ± 1.36*
k_{el} (h^{-1})	0.011 ± 0.003	0.012 ± 0.002	0.015 ± 0.005	0.028 ± 0.009*
$t_{1/2}$ (h)	65.6 ± 16.3	62.2 ± 20.07	50.5 ± 16.0	27.3 ± 10.8**
Cl_p (ml/h)	265 ± 28	264 ± 18	260 ± 28	263 ± 10
Vd (l/kg)	102 ± 33	95 ± 23	77 ± 30	42 ± 18*
AUC_{0-24} (ng ml^{-1} h)	476 ± 46	392 ± 26	493 ± 69*	1001 ± 37***
F %	100	82.4 ± 5.3	51.8 ± 7.2***	21.0 ± 0.8***

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1 **Legends to figures**

2

3 **Figure 1: Structure of sulforaphane transitions monitored in the MRM mode**

4

5 **Figure 2: Determination of sulforaphane in rat plasma**

6

7 A. Representative chromatogram of sulforaphane in spiked plasma extracts at the
8 limit of detection; B. Representative chromatogram of sulforaphane in spiked plasma
9 extracts at the limit of quantification. The outer peak is the more abundant m/z 178 to
10 114 transition whereas the inner peak represents that of m/z 114 to 72.
11 CPS, counts per second

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14 **Figure 3: Plasma concentration vs time curves in rats following oral and**
15 **intravenous exposure to sulforaphane**

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17 Blood samples were withdrawn 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hours after
18 administration. Bars represent SD values where n=4

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