XENOBIOTICA , 1997, VOL. 27, NO. 11, 1113–1120

Determination of the rate of aldicarb sulphoxidation in rat liver, kidney and lung microsomes

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Received 17 April 1997

1. The rate of sulphoxidation of aldicarb (2-methyl-2-(methylthio) propanal *O*-[(methylamino)carbonyloxime], Temik®) in rat hepatic, renal and pulmonary microsomes was determined by quantitating the levels of aldicarb sulphoxide and aldicarb sulphone produced during incubations. Under *in vitro* experimental conditions used in the present study, aldicarb sulphoxide was the only metabolite produced, and further metabolism of aldicarb sulphoxide to aldicarb sulphone was negligible.

2. The average maximal velocity (μmol/min/mg protein) for the sulphoxidation of aldicarb, based on measurements of product formation, in liver, kidney and lung microsomes was 5·41, 39·51 and 2·45 respectively. The corresponding values for the Michaelis constant (μM) were 184, 1050 and 188 respectively.

3. These results imply that under *in vivo* conditions (1) aldicarb sulphoxidation is not likely to be saturable even at lethal doses in the rat, and (2) aldicarb clearance in rat liver and kidney will be limited by the rate of blood flow and not metabolizing enzyme levels.

Introduction

Aldicarb (2-methyl-2-(methylthio) propanal *O*-[(methylamino) carbonyl oxime], Temik®) is widely used to control insects, mites and nematodes (World Health Organization 1991). In mammals, it is readily absorbed and distributed to all tissues by systemic circulation (Knaak *et al*. 1966, Andrawes *et al*. 1967, Dorough *et al*. 1970, Cambon *et al*. 1979). It is initially oxidized to aldicarb sulphoxide (ALX) and subsequently to aldicarb sulphone; aldicarb and its metabolites are susceptible to hydrolysis, with the subsequent dehydration giving rise to the corresponding oximes and nitriles (Baron and Merriam 1988). Although hydrolysis destroys the insecticidal activity, both aldicarb and its oxidative metabolites, ALX and aldicarb sulphone, are potent cholinesterase inhibitors (Hastings *et al*. 1970, Cambon *et al*. 1979, Baron and Merriam 1988).

Whereas the *in vitro* and *in vivo* metabolism of aldicarb has been studied in a variety of mammalian and non-mammalian species and plants (Knaak *et al*. 1996, Metcalf *et al*. 1966, Andrawes *et al*. 1967, Bull *et al*. 1967, Dorough and Ivie 1968, Bartley *et al*. 1970, Dorough *et al*. 1970, Montesissa *et al*. 1991, 1994, 1995), the maximal velocity (V_{max}) and the Michaelis affinity constant (K_m) for aldicarb sulphoxidation have only been determined in fish. Schlenk and Buhler (1991) determined the V_{max} and K_m for aldicarb sulphoxidation in rainbow trout using liver, kidney and gill microsomes. In all three tissues, ALX was the major metabolite, with trace amounts of ALX oxime being formed in the liver, and aldicarb oxime in kidney and liver.

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The information on the quantitative nature (i.e. rate and affinity) of the metabolism of aldicarb is essential to evaluate its biopersistance and profile of elimination in other non-target species such as rodents and man. Accordingly, the objective of the present study was to determine the V_{max} and K_m for aldicarb sulfoxidation in rat liver, kidney and lung microsomes.

Materials and methods

Materials

Aldicarb (ALD), aldicarb sulphoxide (ALX), aldicarb sulphone (ALU) were obtained from Chem Service (West Chester, PA, USA) and were at least 98% pure. The purity of the carbamates was verified by hplc analysis (EPA method 531·1) prior to all experiments. NADPH, Tris-HCl, Tris-acetate, potassium chloride, potassium phosphate, sucrose and EDTA were obtained from Sigma Chemical Co. (St Louis, MO, USA). Methanol (hplc grade), glycerol and sodium pyrophosphate were purchased from Fischer Chemicals (Montréal, Québec, Canada). NaOH and *o*-phthalaldehyde (2-dimethylamino ethanediol hydrochloride, OPA) were purchased from Pickering Laboratories (Mountain View, CA, USA).

Preparation of microsomes

Male Sprague-Dawley rats weighing 180–200 g were obtained from Charles River Canada (St Constant, Québec, Canada) and maintained in stainless steel cages on Purina Certified Rodent Chow (Ralston-Purina Co., Ontario, Canada) and water *ad libitum*. Following a 4–7-day acclimatization, the rats were euthanized (following exposure to CO_2), exsanginated and the tissues (liver, kidney and lung) from individual animals obtained. All tissues were blotted with filter paper (Whatman no. 1), weighed and washed with ice-cold Tris-HCl buffer (0·1 M, pH 7·4) containing 0·1 M KCl and 1 mM EDTA. Liver and kidney tissues from several animals were pooled and homogenized in Tris-HCl buffer (0·1 M, pH 7·4, 1:4 v/v) with a Teflon homogenizer. The tissue homogenates were initially centrifuged at 10000 *g* for 20 min and the supernatant was re-centrifuged at 100000 *g* for 60 min. The resulting pellet was re-suspended in the above buffer and the homogenate centrifuged at 100000 *g* for 60 min. The final pellet was suspended in 0·1 M Tris-HCl containing 0·25 M sucrose and 5 mM EDTA at a volume equal to the weight of the tissue. The same procedure was followed for the preparation of lung microsomes, except that in the second centrifugation 0·1 M potassium pyrophosphate buffer (pH 7·4) was used, and the final pellet was suspended in 0·01 M Tris-acetate buffer containing 1 mM EDTA and 20% (v/v) glycerol (pH 7·4) (Reitz *et al*. 1996). The microsomes were stored at −70 °C and used within 2 months of preparation. The concentration of protein in the was determined immediately after the last centrifugation with the Bio-Rad® method (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, this method involves the incubation of an aliquot of the microsomal preparation with the Bio-Rad Dye reagent (mixture of Coomassie Brilliant Blue, ethanol and phosphoric acid) and the subsequent determination of the optical density of the solution at 595 nm (Bradford 1976).

In vitro assays

The experimental approach consisted of the addition of ALD to a mixture of microsomes, 5 mM NADPH and 0·1 M potassium phosphate buffer (pH 7·4) containing 5 mM EDTA, in a total volume of 1 ml. The optimal NADPH concentration (5 mM) and pH (7·4) were chosen on the basis of preliminary studies with rat liver and/or kidney microsomes (data not shown). The rate of ALD metabolism was assayed by measuring the production of ALX and ALU. In all assays duplicate controls were used as references. In the first one, ALD was incubated with buffer alone and was used to check for contamination and/or non-enzymatic degradation of ALD. In the second control experiment, all components except NADPH were added to the incubation mixture and were used to evaluate the residual metabolic activity of the microsomes. All incubations were conducted in 5-ml glass screw cap tubes at 37 °C.

Time-course assays

The linearity of incubation time was determined by incubating ALD (5·25 or 10·5 μM final concentration, in 20 μl methanol) with 0·14–0·51 mg/ml microsomal protein for up to 60 min (liver and lung 60 min, kidney 45 min). Microsomal protein was added to tubes already containing 5 mM NADPH, and the reaction was initiated with the addition of ALD (in 20 μl methanol). At different time points, the reaction was terminated by adding 0·5 ml methanol and immersion of the assay tubes in ice. All tubes

were centrifuged for 15 min at 3200 *g* (4 °C) to remove the protein precipitate. The supernatant was transferred to 2-ml glass vials sealed with Teflon-coated rubber septa and analysed immediately for levels of ALX.

Protein-course assay

The linear range of microsomal protein concentration was determined by incubating ALD (final concentration 5·25 or 10·5 μM) with various amounts of protein (final concentration 0·06–12 mg/ml) for 10 min and measuring the concentrations of ALX.

Kinetic analyses

The kinetic parameters for ALD sulphoxidation were determined by adding various quantities of ALD (dissolved in 20 μl methanol; final concentrations 36–3700 μM) to a mixture of microsomes (corresponding to 0·18–0·32 mg protein per ml), cofactor (5 mM NADPH) and of 0·1 M potassium phosphate buffer (pH 7·4, 1 ml final volume) at 37 °C and determining the concentration of ALX at the end of a 10-min incubation.

Analytical methods

For the separation and quantitation of ALD and its metabolites, the EPA method 531·1 was used (USEPA 1989). A Varian® hplc system equipped with an autosampler (Model 9100), and a programmable fluorescence detector (Model 9070) linked to a Varian Star LC workstation was used. A dual post-column derivatization system (PCX-5100, Pickering Laboratories, Mountain View, CA, USA) was connected to the hplc system. The post-column reaction unit consisted of two reagent pumps, an hplc column thermostat controlled at 42 °C, and two reaction coils. The first reaction coil was heated to 100 °C for NaOH hydrolysis of ALD, ALX and ALU and the second one was kept at ambient temperature for OPA derivatization of the methyl amine resulting from the hydrolysis of the carbamates.

The separation was achieved with a Pickering C18 column (250 × 4·6 mm i.d., 5-mm packing) placed in the thermostat of the post-column reaction unit and maintained at 42 °C. The mobile phase employed a simple water : methanol gradient. The initial composition was 8% methanol: 92% water, which was maintained for a 1-min hold, after which a 20-min linear gradient program to 20% methanol: 80% water was begun. The mobile phase composition was then changed to 50 : 50 and an 8-min gradient to 80% methanol: 20% water was initiated. Subsequently, the mobile phase was set at 100% methanol for 2 min to provide column clean-up, before returning to the initial condition. The flow rate was 1 ml/min. Under these conditions, ALX elutes first (14·5 min) followed by ALU (16·5 min) and ALD (25·5 min). The separated carbamates were derivatized with OPA to improve sensitivity and selectivity, and the fluorescence of the resulting 1-methylthio-2-methylisoindole was quantified. Both NaOH solution and the OPA reagent in the post-column reaction unit were constantly pumped at a flow rate of 0·3 ml/min during the whole sequential cycle. The injection volume was 10 μl. Excitation and emission wavelengths of the fluorescence detector were set at 330 and 466 nm respectively. Calculations of the concentrations of carbamates in samples were based on area measurement.

Data analysis

The metabolic constants (V_{max} and K_m) for aldicarb sulphoxidation in rat liver, kidney and lung microsomes were determined from Hanes–Woolf plots of the data on ALX concentration obtained at the end of incubation with the corresponding initial concentrations of ALD.

Results

The initial series of studies focused to determine the linear range of incubation time and protein concentration with respect to ALD sulphoxidation in rat tissue microsomes. Figure 1 shows the time-course of ALX formation in rat liver, kidney and lung microsomal preparations for an initial ALD concentration of 5·25 μM (liver and kidney) and 10·5 μM (lung). With the choice of 10 min from the linear part of this curve, the influence of protein concentration on the rate of ALX formation was elucidated. The effect on ALD sulphoxidation was linear for microsomal protein concentrations of up to 1 mg/ml in liver, 0·6 mg/ml in kidney, and 0·4 mg/ml in lung microsomes respectively (Figure 2).

The final series of experiments involved the determination of the rate of ALX

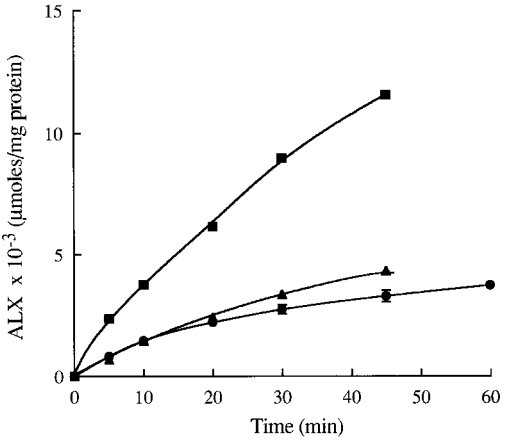

Figure 1. Aldicarb sulphoxide (ALX) produced by the sulphoxidation of aldicarb by rat liver (●, protein concentration 0·5 mg/ml, ALD 5·25 μM), kidney (▲, 0·47 mg/ml, 5·25 μM) and lung (■, 0·14 mg/ml, 10·5 μM) microsomes as a function of incubation time. The symbols represent experimental data (mean ± SE, n = 3).

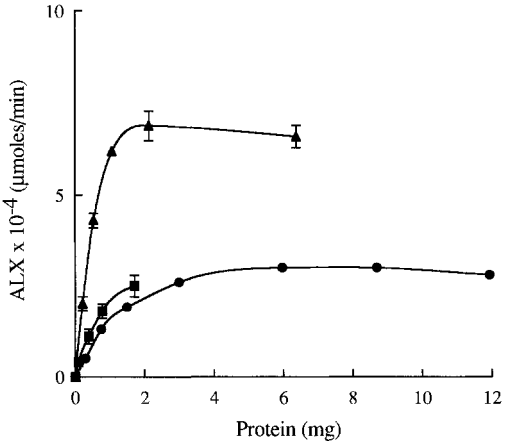

Figure 2. Aldicarb sulphoxide (ALX) produced by the sulphoxidation of aldicarb by rat liver (●, 5·25 μM), kidney (▲, 10·5 μM) and lung (■, 10·5 μM) microsomes as a function of the concentration of microsomal protein. The experimental data (symbols, mean ± SE, n = 3) correspond to the amount of ALX measured at the end of a 10-min incubation.

formation by liver, kidney and lung microsomes following a 10-min incubation with 36–3700 μM ALD (final concentrations). From the measurement and analysis (Hanes–Woolf plot) of ALX concentrations at the end of ALD incubations during this series of experiments, the maximal velocity for metabolism (V_{max}) and Michaelis affinity constant (K_m) for ALD sulphoxidation in rat liver, kidney and lung microsomes were estimated (Figures 3–5). The V_{max} (μmol/min/mg protein) for ALD metabolism in liver, kidney and lung microsomes were 5·41, 39·51 and 2·45 respectively, with the corresponding K_m's (μM) being 184, 1050 and 188. Under the experimental condition of the present study, (1) incubation of ALD with liver, kidney and lung microsomes resulted exclusively in the formation of ALX, and (2) the oxidation of ALX to ALU by either liver, kidney or lung microsomes was negligible (data not shown).

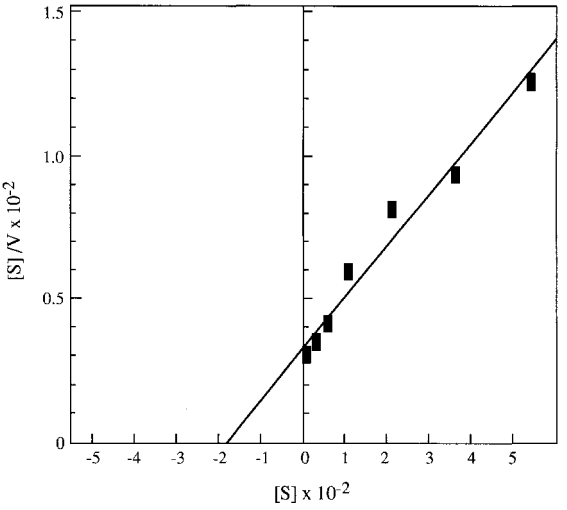

Figure 3. Hanes–Woolf plot of aldicarb sulphoxidation in rat liver microsomes. v, initial rate of reaction (μmol/min/mg protein); [S], initial aldicarb concentration (μM).

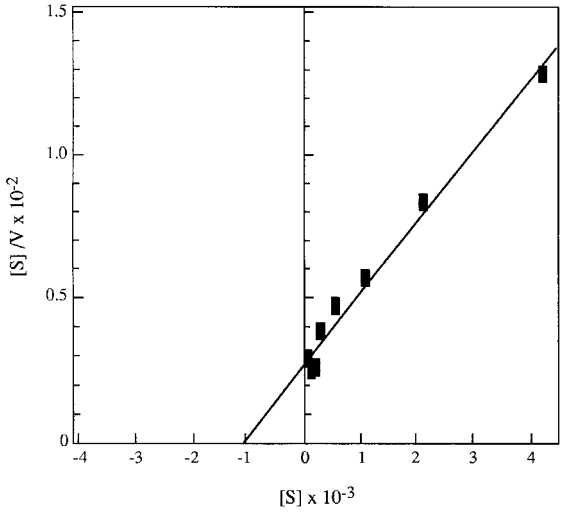

Figure 4. Hanes–Woolf plot of aldicarb metabolism in rat kidney microsomes.

Discussion

Aldicarb sulphoxidation is considered to be a bioactivation process since the primary oxidative metabolite (ALX) is more potent than the parent chemical (ALD) as an acetylcholinesterase inhibitor (World Health Organization 1991). The *in vitro* metabolism of ALD has been investigated using subcellular fractions or whole cells isolated from rat, rabbit, sheep, cattle, goat, chicken and fish (Andrawes *et al*. 1967, Montesissa *et al*. 1991, 1994, 1995, Schlenk and Buhler 1991, Venkatesh *et al*. 1991). All of these studies except that of Schlenk and Buhler (1991) and Venkatesh *et al*. (1991) are at best semiquantitative in nature. In general, these latter studies have shown that (1) ALX is the major product of ALD sulphoxidation and (2) ALD sulphoxidation could be mediated both by cytochrome P450 and flavin-containing monooxygenases (FMO). The experimental designs used in these latter studies

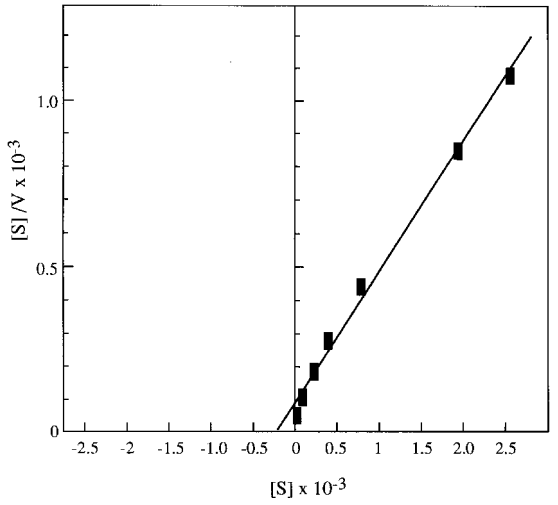

Figure 5. Hanes–Woolf plot of aldicarb metabolism in rat lung microsomes.

could additional provide a qualitative characterization of the profile of metabolites found at the end of incubation, but not quantitative information (V_{max}, K_m) on ALD metabolism. Schlenk and Buhler (1991) and Venkatesh *et al.* (1991) on the other hand reported the V_{max} and K_m for ALD sulphoxidation using microsomes from fish organs and purified renal and hepatic FMO from mouse respectively. Since such quantitative information on ALD oxidation in rat tissues is not available in the literature, the present study estimated the affinity and maximal velocity of ALD sulphoxidation using microsomes isolated from rat liver, kidney and lung.

The Michaelis affinity constant for ALD oxidation in rat liver and kidney microsomes are comparable with those reported by Venkatesh *et al.* (1991) using purified FMO from mouse tissues. Regardless of the preparation and species, the affinity constant for metabolism of a substrate is anticipated to be the same, as long as the same isoenzyme(s) is involved. This has formed the very basis of some, current default approaches for *in vitro* to *in vivo* and interspecies extrapolations of xenobiotic metabolism (Krishnan and Andersen 1994). The fact that the K_m's estimated in the present study are comparable with those reported by Venkatesh *et al.* (1991) (liver 196 μM, kidney 385 μM) adds further support to the preceding practice.

The results of the present study indicate that the K_m for ALD sulphoxidation is comparable in liver and lung (184 versus 188 μM). Such a similarity in K_m for the sulphoxidation of several FMO substrates has been reported previously (Venkatesh *et al.* 1991). Based on the K_m obtained in the present study, it may be suggested that ALD oxidation is not saturable even at lethal doses in the rat ($LD_{50} \approx 1$ mg/kg; World Health Organization 1991). Therefore, the rate of ALD oxidation in rat liver, kidney and lung can be described as a first-order process. The intrinsic clearance values (V_{max}/K_m) for ALD sulphoxidation in rat liver, kidney and lung are 7·06, 1·02 and 0·051 l·min^{-1} respectively. For the first-order conditions, the clearance of ALD in each of these tissues can be calculated as:

$$\frac{[V_{max}\ (\text{mg/min})/K_m\ (\text{mg/l})]*Q_t\ (\text{l/min})}{[V_{max}\ (\text{mg/min})/K_m\ (\text{mg/l})]+Q_t(\text{l/min})},$$

where Q_t is the rate of blood flow to tissue t (liver = 0·016 l/min, kidney = 0·013 l/min, lung = 0·090 l/min) (ILSI 1994). Since the value of (V_{max}/K_m) is very large with respect to Q_t in rat liver and kidney, Q_t in the denominator of the above equation becomes negligible, making organ clearance of ALD equal to Q_t. The pulmonary clearance of ALD, however, is not limited solely by Q_t. Therefore, in the case of lung, both intrinsic clearance parameters and Q_t are critical determinants of ALD clearance. This is principally due to the fact that Q_t for lung is very large (i.e. equal to cardiac output), and the volume of the lungs is small relative to other metabolizing tissues. Given that the volume of liver is greater than that of kidney and lung, the former is likely to be the most important tissue metabolizing ALD in the rat. The rate of enzymatic sulphoxidation of ALD in rat organs, and its dependence on blood flow rates, elucidated in the present study, have important implications for predicting the *in vivo* kinetics of ALD in the rat and subsequent extrapolation to man for risk assessment purposes.

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