Toxicology Letters 224 (2014) 290–299

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Comparative metabolism of benfuracarb in *in vitro* mammalian hepatic microsomes model and its implications for chemical risk assessment

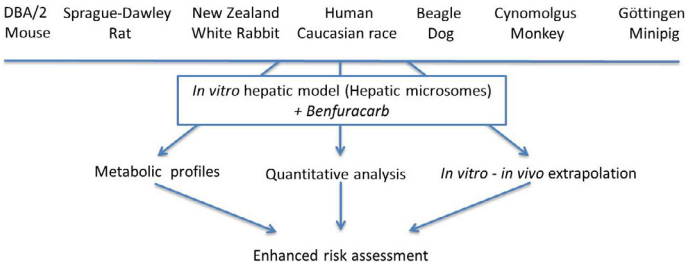
Khaled Abass[a,b,*,1], Petri Reponen[b,c], Sampo Mattila[c], Arja Rautio[a], Olavi Pelkonen[b]

[a] *Center for Arctic Medicine, Thule Institute, University of Oulu, Finland*
[b] *Pharmacology and Toxicology Unit, Institute of Biomedicine, P.O. Box 5000, FI-90014 University of Oulu, Oulu, Finland*
[c] *Department of Chemistry, P.O. Box 3000, FI-90014 University of Oulu, Finland*

HIGHLIGHTS

- We studied benfuracarb species-specific quantitative metabolic profiles/activities.
- A total of seven phase I metabolites were detected from the extracted chromatograms.
- Quantitative interspecies differences in the metabolic profiles/kinetics were observed.
- *In vitro* studies are helpful for the proper selection/interpretation of animal models.
- Our findings provide valuable information for chemical risk assessment.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 May 2013
Accepted 7 August 2013
Available online 16 August 2013

Keywords:
Pesticides
Toxicokinetics
P450
In vitro metabolism
Risk assessment

ABSTRACT

In vitro metabolism of benfuracarb in liver microsomes from seven species was studied in order to quantitate species-specific metabolic profiles and enhance benfuracarb risk assessment by interspecies comparisons. Using LC–MS/MS, a total of seven phase-I-metabolites were detected from the extracted chromatograms and six of them were unequivocally identified. Benfuracarb was metabolized *via* two metabolic pathways, the sulfur oxidation pathway and nitrogen sulfur bond cleavage, yielding carbofuran, which metabolized further. Analysis of the metabolic profiles showed that benfuracarb was extensively metabolized with roughly similar profiles in different species *in vitro*. *In vitro* intrinsic clearance rates as well as calculated *in vivo* hepatic clearances indicated that all seven species metabolize benfuracarb *via* the carbofuran metabolic pathway more rapidly than the sulfoxidation pathway. The highest interspecies differences in hepatic clearance rate values were for mouse and rat liver microsomes compared to human, *i.e.* 4.8 and 4.1-fold higher, as illustrated by *in vivo* hepatic clearance of carbofuran. Overall, there are quantitative interspecies differences in the metabolic profiles and kinetics of benfuracarb biotransformation. These findings illustrate that *in vitro* studies of benfuracarb metabolite profiles and toxicokinetics are helpful for the proper selection and interpretation of animal models for toxicological evaluation and chemical risk assessment.

* Corresponding author at: Center for Arctic Medicine, Thule Institute, P.O. Box 7300, FI-90014 University of Oulu, Oulu, Finland. Tel.: +358 294 483563.
E-mail addresses: khaled.megahed@oulu.fi, khaled.m.abass@gmail.com (K. Abass).
[1] Permanent address: Department of Pesticides, Menoufia University, P.O.Box 35211, Egypt.

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http://dx.doi.org/10.1016/j.toxlet.2013.08.009

1. Introduction

Benfuracarb (CAS RN. 82560-54-1) has insecticidal activity against a number of insects in rice, corn, beet, and other agricultural crops. Benfuracarb as well as its main metabolite, carbofuran, act as substrates for acetylcholinesterase and initially form a Michaelis-like reversible complex. The complex decarbamoylation is so slow that the enzyme is effectively irreversibly inhibited leading to neurotoxic effects (Roberts and Hutson, 1999).

Absorption and translocation of benfuracarb has been examined in the bean and corn plants, and it was initially converted into carbofuran, which was subsequently oxidized at the 3-position of the ring and the N-methyl moiety (Tanaka et al., 1985; Umetsu et al., 1985). In the housefly, benfuracarb was rapidly biotransformed to carbofuran, and consequently to 3-hydroxy carbofuran, as major metabolites. Minor metabolites were N-hydroxylmethyl-carbofuran, 3-keto-carbofuran, 3-hydroxy-carbofuran phenol, 3-hydroxy-N-hydroxymethyl-carbofuran, 3-keto-N-hydroxymethyl carbofuran, and several minor unidentified products (Usui and Umetsu, 1986). In mammals, benfuracarb has been shown to metabolize rapidly and is almost completely excreted in the urine and feces of rats within 7 days. Major metabolites found in feces were carbofuran, carbofuran phenol, 3-hydroxy carbofuran, 3-hydroxy phenol, and 3-ketophenol. These major metabolites were eliminated into the urine as β-glucuronide conjugates (Roberts and Hutson, 1999). In human, mouse, and rat liver microsomes two pathways, carbofuran formation and benfuracarb sulfoxide formation, have been detected by an undescribed analytical technique (Chang et al., 2008).

Different analytical methods have been applied for unchanged benfuracarb detection in different matrices. LC–MS (Chung and Chan, 2010; Lee et al., 2011), LC–UV (Valenzuela et al., 1999), and GC–MS (Chu et al., 2005; Nguyen et al., 2010) were used in fruit and vegetable extracts, while LC–MS as well as GC–MS were used in animal tissues (Pang et al., 2006). On the other hand, benfuracarb and its main metabolite carbofuran were detected using TLC and GC/MS in human blood (Lee et al., 1999). In our study, the advantages of LC/TOF-MS and LC/MS–MS for metabolite identification and quantification were applied to the study of benfuracarb biotransformation in a mammalian hepatic *in vitro* model.

Benfuracarb and its main metabolite, carbofuran, are both active substances/compounds. Acute toxicity of carbofuran is 25–27-fold that of benfuracarb in rats (Tomlin, 2000). Thus, the comprehensive metabolism of benfuracarb is also important to elucidate. Cytochrome P450 enzymes (CYP) are also important in the metabolism of pesticides, especially insecticides. A database containing a comprehensive literature survey on the role of CYP enzymes in pesticide biotransformation *in vitro* and the types of reactions mediated has recently been published (Abass et al., 2012).

The CYP activities exhibit a wide range of variability between mammalian species (Turpeinen et al., 2007). In addition to toxicological data, *in vitro* toxicokinetic data for the active chemical moiety will be valuable in further developing the proposed default subdivision of the usual uncertainty factor to quantitative toxicokinetic chemical-specific assessment factors (CSAFs) (WHO/IPCS, 2005). Moreover, the characterization of metabolic factors and toxicokinetics will enhance benfuracarb risk assessment by providing quantitative toxicokinetic data to make reliable comparisons between species (Paine, 1996; Falk-Filipsson et al., 2007). Therefore, the identification and quantification of benfuracarb metabolites produced by several mammalian species was investigated in an *in vitro* hepatic model by LC/MS–MS to examine interspecies differences and its implications for chemical risk assessment.

2. Materials and methods

2.1. Chemicals

Benfuracarb and its metabolites were purchased from ChemService (West Chester, PA). Dibutylamine was purchased from Sigma–Aldrich (Germany). HPLC-grade solvents were obtained from Rathburn (Walkerburn, UK) and Labscan (Dublin, Ireland). All other chemicals used were from the Sigma Chemical Company (St. Louis, MO) and were of the highest purity available. Water was freshly prepared in-house with the Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was UP-grade (18.2 MΩ).

2.2. Human liver homogenates and mammalian liver microsomes

Human liver samples used in this study were obtained from the University Hospital of Oulu as surplus from organ donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. All liver samples were of Caucasian race including 4 female and 6 male between the ages of 21 and 62. Detailed characteristics of the liver samples are presented in our previous publication (Abass et al., 2007). The livers were transferred to ice immediately after the surgical excision and cut into pieces, snap-frozen in liquid nitrogen, and stored at −80 °C. Human liver homogenate was prepared from livers of ten individuals by homogenizing liver tissue in four volumes of 0.1 M phosphate buffer (pH 7.4), *i.e.* the homogenate contained 200 mg of hepatic tissue per ml. Male DBA/2 mouse, Sprague-Dawley rat, Beagle dog, Cynomolgus monkey, Göttingen minipig, and New Zealand white rabbit liver samples were obtained after approval of the Ethics Committee of the University of Oulu, Finland. All microsomes were prepared by standard differential ultracentrifugation (Pelkonen et al., 1974). The final microsomal pellet was suspended in 100 mM phosphate buffer, pH 7.4. Protein content was determined by the Bradford method (Bradford, 1976).

2.3. In vitro assay of benfuracarb metabolites

The standard incubation mixture contained 100 μM benfuracarb, 0.15 mg pooled liver microsomal protein, and 1 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). Benfuracarb was prepared once a week in dimethylsulfoxide (DMSO; final amount in the reaction medium 1.0%). After a 2-min incubation at +37 °C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany), the reaction was started by adding NADPH. The mixture was incubated at +37 °C for 20 and 60 min and the reaction was stopped with 600 μl of ice-cold acetonitrile containing an internal standard. All incubations were carried out in triplicate. After centrifugation at 10,000 × *g* for 15 min, the supernatant was collected and stored at −20 °C until analyzed.

To measure the production of potential metabolites, human liver homogenate incubations were prepared containing the same final benfuracarb concentration as the microsomal incubations. In addition to 40 μl of human liver homogenate (contains approximately 0.14 mg microsomal protein), the other components in homogenate incubates were 5 mM uridine 5′-diphosphoglucuronic acid (UDPGA), 1 mM glutathione, 1.2 mM adenosine-3′-phosphate-5′-phosphosulfate (PAPS), and 1 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). The mixture was incubated at +37 °C for 20 and 60 min and the reaction was stopped with 600 μl of ice-cold acetonitrile containing an internal standard. The analytical method was similar to the microsomal preparations. Results are expressed as a mean ± standard deviation for three replicates.

2.4. Kinetic parameters

To measure the enzyme kinetic parameters in microsomal samples, the standard incubation mixture contained benfuracarb (2.5–300 μM). Incubation mixtures and methods were the same as mentioned above, except the incubation times were 20 min for microsomal samples. Samples were analyzed by LC–MS–MS. The kinetic parameters V_{max} and K_m were calculated using Prism 5.0 (GraphPad Software, Inc., San Diego, CA) by nonlinear regression. These values were used to calculate the intrinsic clearance value (V_{max}/K_m). All results are expressed as mean ± standard error for three replicates. In the standard experimental conditions used for benfuracarb metabolites, reaction rates were linear at least up to 0.15 mg of microsomal protein/ml and 60 min of incubation time.

2.5. Chromatography of the benfuracarb metabolites

Samples were centrifuged before analysis for 10 min at 10,000 × *g*. Chromatographic separation was carried out with the Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA). The column used was a Waters Atlantis T3 (2.1 mm × 100 mm, particle size of 3 μm) together with a Phenomenex C18 2.0 mm × 4.0 mm precolumn (Phenomenex, Torrance, CA). The temperature of the column oven was 45 °C. The eluent flow rate was 0.4 ml/min. The eluents used were ultrapure-grade water containing 0.1% acetic acid (A) and methanol (B). A linear gradient elution from 5% B to 75% B in 8 min was applied. Solvent B was thus maintained at 98% for 3 min before re-equilibration (6 min). The total analysis time was 17 min.