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Carboxanilide Persistence in Wildlife: Excretion and Retention in a Rat Model

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Carboxylic acid anilides, and particularly 2-substituted derivatives of the benzanilide (*N*-phenylbenzamide, *N*-benzoylaniline) structure, are widely used as plant growth regulators, antimicrobials and fungicides in the protection of cereals and other crop plants (Courtier and Moreau 1970; Osieka et al. 1968; Pommer 1968; Pommer and Kradel 1969; Schwartz and Skaptason 1966; Woodcock 1977). As with any group of chemicals that are extensively distributed over large areas of the environment, concerns regarding their potential persistence within the biosphere and particularly their accumulation within living creatures are paramount. Potential toxicological issues arise as these compounds are known to inhibit key enzymic processes. They function as fungicides by blocking the succinic dehydrogenase complex involved within the tricarboxylic acid cycle (Pommer 1968). Benzanilide itself inhibits cytochrome oxidase activity in a variety of tissues and its 2-hydroxyderivative, salicylanilide, uncouples oxidative phosphorylation in hepatocyte and chondrocyte mitochondria (Abood and Gerard 1953; Whitehouse 1964). Any attempts at the estimation of persistence of these compounds within animals necessarily requires the measurement and inclusion of all metabolic products, whether known or not, and would therefore be greatly aided by the incorporation of a readily quantifiable and permanent marker within the parent molecule. Employing the rat as a model, the present paper describes the persistence of benzanilide and eight of its 2-substituted derivatives following the single oral administration of a known dose of each [^{14}C]-radiolabelled benzanilide.

MATERIALS AND METHODS

Benz-[U-^{14}C]-anilide was prepared by reacting [U-^{14}C]-aniline (Nycomed-Amersham, Amersham, UK) with benzoyl chloride (Sigma-Aldrich, Gillingham, Kent, UK) under Schotten-Baumann conditions (Baumann 1886; Schotten 1884; Sontag 1953). The use of appropriately 2-substituted benzoyl chlorides (Sigma-Aldrich) permitted the synthesis of the 2-substituted benzanilides with the exception of salicyl-[U-^{14}C]-anilide that was prepared by refluxing [U-^{14}C]-aniline with salicylic acid in the presence of phosphorus trichloride (Wanstraat 1873). The products were collected by suction filtration and recrystallised several times from hot ethanol to give crystalline material that was dried *in vacuo* at 40ºC

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for 12 hours before use. All products (sp. act. 12-15 mCi/mol) were pure (both chemically and radiochemically; >99%) by either thin-layer (tlc), gas-liquid (gc) or high-pressure liquid (hplc) chromatography, gave elemental analyses (CHN) and mass spectral fragmentation in agreement with expected theoretical and had melting points corresponding to literature values. All compounds were shown to be free of aniline to a limit of 0.002% by wt (20 ppm).

Nine groups of four male rats (250-300 g wt, aged 26 wk, Wistar strain; Harlan OLAC, Bicester, Oxfordshire, UK) were administered a single dose of a triturated [^{14}C]-benzanilide (150 mg/kg body weight) via gastric intubation as an aqueous suspension containing bile salts (sodium tauroglycocholeate from ox bile, 10 g/L; Sigma-Aldrich), and kept individually in metabolism cages (Metabowls; Jencons Ltd, Herts, UK) which permitted the separate collection of urine and feces. At the end of the experiment the animals were euthanaised by carbon dioxide asphyxiation.

Aliquots of urine (0.1 to 1.0 mL) were added directly to a scintillation fluid ('Ecoscint'; National Diagnostics Ltd, New Jersey, USA) and counted by liquid scintillation spectrometry (Packard Tricarb 4640, Ambac Industries Inc, Illinois, USA), internal standards being used for quench correction. Feces were lyophilized, ground to a powder and triplicate samples (*c.* 100 mg) combusted in oxygen (Harvey Biological Material Oxidiser, ICN Tracer Labs Ltd, Horsham, West Sussex, UK), the $^{14}CO_2$ so produced being trapped in an alkaline scintillation mixture and counted as above. Before processing the carcasses, the gastrointestinal tracts were tied above the stomach and below the rectum and removed by dissection. The remains of the carcasses for the four animals within each group were pooled, as were the separated gastriointestinal tracts, and they were then digested by submersion in aqueous potassium hydroxide (4 M, 2.5 litre) at 37°C for 10 d. The resultant liquid was filtered through glass wool, neutralized with HCl and aliquots (1.0 mL) in scintillation vials were decolourized with hydrogen peroxide (30% by wt, 2 ml), methanol (0.5 mL) being added to prevent effervescence. Following heating (60-70°C, 30 min) to remove excess peroxide, scintillation fluid was added, the vial vortex mixed and permitted to stand for 48 hr in the dark to allow decay of any chemiluminescence before being counted as above.

RESULTS AND DISCUSSION

Throughout the duration of the study no gross toxicity was observed in any animal following the oral administration of the nine benzanilide compounds. The dose level employed was in excess of that likely to be encountered in the environment but would enable any persistence of the compound or its metabolites within the animals to be clearly detected. The radioactive mass balance studies achieved good overall recoveries with little interindividual variation being seen between the animals within each study group (Table 1). From data obtained during the sequential collection of urine, an estimate of the role played by the kidney in the removal of these compounds can be made. Graphical plotting gave a value of

10.6±3.3 hr (mean ± s.d.; range 6.6 to 15.9 hr) for the urinary half-life ($t_{½\beta}$), the mean value being close to that obtained for the parent compound, benzanilide (9.6 hr) (Rowland and Tozer 1995). However, the overall picture is more complicated than this with certain substituted benzanilides being less readily absorbed than others from the gastrointestinal tract or being more efficiently excreted via the biliary system. Both of these factor enabling larger amounts to be excreted within the feces.

Table 1. Percentage administered radioactivity excreted in urine and feces over 0-3 days by groups of male rats (mean ± s.d., n = 4).

[^{14}C]-Compound		day 1	day 2	day 3	Total
Benzanilide	urine	62.3±2.9	13.1±1.2	1.2±0.9	76.6
	faeces	14.1±1.7	6.0±1.8	0.6±0.1	20.7
					97.3
2-Bromobenzanilide	urine	29.9±9.9	4.1±1.3	0.8±0.4	34.8
	faeces	35.2±2.9	5.8±0.5	1.0±0.6	42.0
					76.8
2-Chlorobenzanilide	urine	53.6±5.2	3.3±1.6	1.0±0.6	57.9
	faeces	15.5±5.6	5.8±2.6	3.7±2.4	25.0
					82.9
2-Fluorobenzanilide	urine	75.9±6.6	2.4±0.8	0.9±0.3	79.2
	faeces	6.8±1.3	1.8±0.8	0.8±0.4	9.4
					88.6
2-Hydroxybenzanilide	urine	62.0±7.4	10.1±2.3	1.9±0.2	74.0
	faeces	16.5±2.8	3.6±0.3	0.6±0.1	20.7
					94.7
2-Iodobenzanilide	urine	10.9±2.6	4.2±0.5	1.1±0.3	16.2
	faeces	58.9±4.1	16.0±2.2	2.1±0.5	77.0
					93.2
2-Methylbenzanilide	urine	57.8±2.4	7.6±1.6	1.1±0.3	66.5
	faeces	13.3±2.8	3.1±2.0	0.5±0.1	16.9
					83.4
2-Methoxybenzanilide	urine	31.5±3.9	6.3±1.0	2.2±0.3	40.0
	faeces	31.1±3.4	10.0±2.2	3.5±1.2	44.6
					84.6
2-Nitrobenzanilide	urine	23.7±2.2	8.2±0.7	2.3±0.3	34.2
	faeces	36.4±5.2	11.5±4.3	1.8±0.6	49.7
					83.9

Nevertheless, although some variation was observed, a considerable amount of the oral dose of these compounds was excreted and removed from these animals within three days (88.4±6.9%). That remaining, especially for bromobenzanilide, was found almost exclusively within the contents of the gastrointestinal tract, presumably awaiting excretion, and was not evident to any significant levels within tissues or organs (carcass), indicating that these compounds were not retained specifically (Table 2).

Table 2. Percentage administered radioactivity detected in male rats after 3 days following administration of the various 2-substituted [^{14}C]-benzanilides.

[^{14}C]-Compound	Percentage dose within carcass			Total recovery (with excreta)
	g.i.tract	remainder	total	
Benzanilide	2.5	0.8	3.3	100.6
2-Bromobenzanilide	23.8	1.5	25.3	102.1
2-Chlorobenzanilide	15.9	0.8	16.7	99.6
2-Fluorobenzanilide	9.8	2.2	12.0	100.6
2-Hydroxybenzanilide	2.3	2.8	5.1	99.8
2-Iodobenzanilide	6.0	1.0	7.0	100.2
2-Methylbenzanilide	13.1	2.8	15.9	99.3
2-Methoxybenzanilide	12.2	1.2	13.4	98.0
2-Nitrobenzanilide	10.9	4.4	15.3	99.2

Although similar studies concerning 2-substituted benzanilides are scarce within the literature, previous data reported for 2-iodobenzanilide (benodanil) has suggested that over 5 days some 96.5% of an oral dose was excreted with only 3.5% remained within the carcass and detailed tissue distribution studies undertaken for this compound confirmed that the majority of the radioactivity within the carcass was indeed present in the gastrointestinal tract (Mitchell and Waring 1981). This supports the assumption that the amounts found remaining within the animals in the present study were on their way to be excreted and not retained within the tissues.

It is usually assumed that the ability to predict a chemical's behaviour in environmental or biological systems is enhanced by knowledge of its physicochemical characteristics, especially its solubility in aqueous and lipid surroundings and its relative partitioning. Although data are scarce, the

benzanilides are generally described as insoluble or sparingly soluble in water and a recent study concerning 14 tri- and/or tetra-substituted benzanilides reported aqueous solubility values of 6.6±2.6 mg/L (range 2.2 to 10.2 mg/L) (Dai et al. 1998). This is why such pesticides need to be formulated with wetting agents. Octanol/water partition coefficients have been quoted for benzanilide (log P 2.62) and salicylanilide (log P 3.27) (Leo et al. 1971) and the 14 substituted benzanilides mentioned above (range, log P 1.83 to 2.64) (Dai et al. 1998), indicating that they are more soluble in lipid than water. This implies that these benzanilides should partition readily into the lipid phases of living organisms. However, it must be appreciated that this modest lipid solubility is several orders of magnitude less than that found for the common organochlorine pollutants. Also, once entry has been gained, the enzymes present within cells will modify the structure of these compounds and vastly increase their aqueous solubility. Hydroxylation at the 4'-position on the aniline ring (para- to the nitrogen), with smaller amounts of 2'-hydroxylation (ortho- to the nitrogen), and subsequent conjugation with glucuronic acid appears to be the metabolic pathway of choice for 2-substituted benzanilides (Mitchell and Waring 1981). Aromatic hydroxylation is a metabolic reaction that occurs within many living organisms including mammals, birds, amphibia and insects. Amongst the mammals, the orientation of aromatic hydroxylation of aniline shows a species variation, with cats, dogs and other carnivores producing mainly the 2-hydroxylated metabolite whereas rabbits and other herbivores produce mainly the 4-hydroxyderivative. Nevertheless, the overall amount of hydroxylated product is surprisingly similar. Glucuronic acid conjugation is a reaction undertaken by most mammals, birds, reptiles and amphibia, but not fish. However, the cat is unusual in that it forms glucuronides with difficulty (Parke 1968).

Consequently, it may be concluded with a reasonable degree of certainty, that even if 2-substituted benzanilides were to gain entry they would not be persistent within mammalian systems.

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