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THE FATE OF MONOCHLOROBENZANILIDES IN THE RAT

S.C. Mitchell[1], J. O'Gorman[1] and R.H. Waring[2]

[1]*Department of Pharmacology and Toxicology, St. Mary's Hospital Medical School, London W2 1PG and* [2]*School of Biochemistry, University of Birmingham, P.O. Box 363, Edgbaston, Birmingham B15 2TT, England*

ABSTRACT

The fate of the three isomeric benzoic acid-substituted monochlorobenzanilides has been studied in the rat after their oral administration. The excretory profile of all three isomers was similar with over half of the dose being excreted in the 0-24 hour urine and between 83-87% being recovered during the three days following administration. The most prominent route of metabolism was that of 4'-hydroxylation on the unsubstituted aniline moiety followed by glucuronic acid conjugation.

INTRODUCTION

The halogenobenzanilides are a group of aromatic compounds which possess antifungal activity and are particularly effective in controlling the various "rust" diseases of cereal plants and other infections caused by the *Basidiomycetes* fungi. Their mode of action is thought to be via an inhibitory effect on the mitochondrial succinate dehydrogenase complex in susceptible fungal strains /1,2/.

These compounds are degraded by microbes within the soil giving pesticides of relatively short environmental persistence /3/. However, fungicides remaining on crops will eventually be encountered by the mammalian system, albeit in small but nevertheless repeated amounts. Although the potential mammalian toxicity of the halogenobenzanilides is unknown, the parent compound, benzanilide, and its 2-hydroxy derivative, salicylanilide, have been shown to inhibit cytochrome oxidase activity in rat heart mitochondria /4/ and to uncouple oxidative phosphorylation in rat liver and cartilage mitochondria /5/, respectively. The longer a compound remains in contact with a living system the greater is its prospect of biochemical interaction which may result in toxicological consequences. As little information is available regarding the passage of halogenobenzanilides through mammalian systems, the retention of three isomeric chlorobenzanilides (Fig. 1) has been examined in the rat following oral administration.

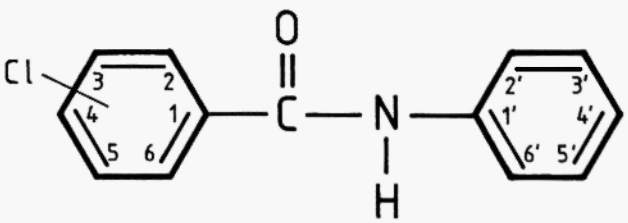

Fig. 1: Chemical structure of monochlorobenzanilide

MATERIALS AND METHODS

Chemicals

The three monosubstituted [U-^{14}C]-chlorobenzanilides were prepared under non-aqueous conditions by a modification of the Schotten-Baumann acylation reaction. [U-^{14}C]-Aniline (Amersham International p.l.c., England) (derived from [U-^{14}C]-aniline hydrogen sulphate by chloroform extraction of a basic solution) was diluted with triple distilled aniline (1.5 ml; 16.5 mmol) and allowed to react with the appropriate chlorine-substituted benzoyl chloride (8 mmol) in dry chloroform. The white precipitate of [U-^{14}C]-aniline hydrochloride formed was removed by filtration and recycled. The chloroform solvent was removed by distillation (b.p. 60-62°C) and the residue allowed to crystallise. Repeated recrystallisation from aqueous ethanol was performed to remove any unchanged acid chloride (by esterification) and the product dried to constant weight over phosphorus pentoxide.

The chlorobenzanilides were obtained as white needle-like crystals in 50% theoretical yields (relative to 8 mmol chlorobenzoyl chloride) with specific activities of 13-15 μCi/mmol and radiochemical purities >98%. The compounds were shown to be chemically pure by t.l.c., mass spectrometry and chemical analysis and had melting points corresponding to literature values (2-chlorobenzanilide, m.p. 115-117°C; 3-chlorobenzanilide, m.p. 122-125°C; 4-chlorobenzanilide, m.p. 193-195°C) /6/. These analytical techniques have been previously described in detail /7,8/.

The 2'-hydroxy, 3'-hydroxy and 4'-hydroxy derivatives of 2-chlorobenzanilide were purchased from Aldrich Chemical Co. Ltd., England and recrystallised from aqueous ethanol. β-Glucuronidase (*E. coli*) and sodium choleate (ox bile) were obtained from Sigma Chemical Co. Ltd., England.

Animals

Male rats (300±20 g body weight; Wistar strain) were given a single oral dose of one of the three triturated chlorobenzanilides (150 mg/kg body wt.; *c.* 2.5 (2.36 -3.11) μCi) via gastric intubation as an aqueous suspension containing sodium choleate (10 g/l), and kept in metabolism cages ("Metabowls", Jencons Ltd., Herts., England) which permitted the separate and fractional collection of urine and faeces.

Further studies were undertaken in animals receiving 2-chlorobenzanilide. Anaesthesia was induced and maintained in male rats by intraperitoneal injection of sodium pentobarbitone (42 mg/kg body weight; "Sagatal", May & Baker Ltd., Essex, U.K.). The common bile duct was catheterised through a ventral midline incision just caudal to the xiphoid cartilage and the catheter taken subcutaneously to emerge at the lower left inguinal region of the abdomen. The animals were allowed to recover in a restraining cage, with free access to food and water, before dosing and collection of bile in which radioactivity was measured as described below. In other animals, samples of blood were removed from the tail vein at known intervals after dosing, immediately weighed and combusted as described below.

Measurement of radioactivity

Aliquots of urine (1.0 ml), bile (0.2 ml) and areas of t.l.c. plates were added directly to a toluene-based scintillation fluid mixed with Triton X-100 (2:1 v/v) /9/ and counted by liquid scintillation spectrometry using a Packard Tri-Carb 4640 scintillation counter (Packard Instruments Ltd., Berks., U.K.) with external standards being employed for quench correction. Faeces were homogenised, lyophilised and ground to a fine powder. Blood samples and powdered faeces (*c.* 100 mg) were combusted in oxygen (Harvey Biological Material Oxidiser, ICN Tracer Lab. Ltd., Horsham, West Sussex); the [^{14}C]-carbon dioxide produced being trapped in a scintillation cocktail containing 2-phenylethylamine /10/ and counted as above.

Chromatography

Thin-layer chromatography (t.l.c.) was carried out on plates coated with silica gel G (0.2 mm thick, 20 x 20 cm, aluminium backed; Merck Darmstadt, FR Germany) using toluene/acetone (9/1 by vol). Compounds were detected by using aqueous tripotassium hexacyanoferrate/iron trichloride (50 g/l) /11/ or diazotised 4-nitroaniline for phenols /12/, or overspraying with concentrated sulphuric acid followed by charring via direct exposure of the silica to a Bunsen flame as a non-specific detection reagent /7/. Glucuronic acid conjugates were detected using naphthalene-1,3-diol (20 g/l) in aqueous trichloracetic acid (330 g/l), followed by heating at 135°C for 5 to 10 minutes /13/. Urine samples, and eluates (phosphate buffer) of undeveloped t.l.c. plate sections, corresponding to previously identified glucuronide-

positive areas (on other plates), were incubated with β-glucuronidase (1,000 units) in 0.1 M potassium phosphate buffer (pH 6.8) for up to 18 h at 37°C. The resultant mixtures were either used neat or extracted with diethyl ether, concentrated by evaporation under nitrogen, and then chromatographed as described above /7,8/. Radioactive areas were located and quantified by cutting the t.l.c. plates into 0.5 cm strips and counting as described for urine samples above.

RESULTS

The results of the determination of radioactivity in the excreta from male rats given a single oral dose of [^{14}C]-chlorobenzanilide (150 mg/kg body weight) are presented in Table 1. Around 60% of the dose was excreted in the first 0-24 hour urine, and during the three days after dosing 83% to 87% of the dose was recovered, a

TABLE 1

Excretion of radioactivity following oral administration of [^{14}C]-chlorobenzanilides to the adult male rat

	2-chloro-benzanilide	3-chloro-benzanilide	4-chloro-benzanilide
Urine			
0-24 h	53.9 ± 5.4 (9)	59.7 ± 5.0 (4)	55.3 ± 6.0 (4)
24-48 h	3.1 ± 1.7 (5)	2.5 ± 1.1 (4)	4.2 ± 0.5 (2)
48-72 h	1.0 ± 0.6 (5)	0.4 ± 0.2 (4)	1.0 ± 0.2 (2)
TOTAL	58.0	62.6	60.5
Faeces			
0-72 h	24.7 ± 2.5 (5)	22.3 ± 4.5 (4)	26.5 ± 6.2 (4)
GRAND TOTAL	82.7	84.9	87.0

Values are expressed as a percentage of the administered radioactive dose (mean ± 1 s.d.)
The number of animals is denoted in brackets.

considerable proportion of this amount (one third) being found in the faeces. Little difference was found in the excretory profiles of the three positional isomers.

Blood radioactivity levels after oral administration of 2-chlorobenzanilide showed a slow rise to an initial peak at around 2 hours, accounting for about 4% of the administered dose (assuming equal distribution and that blood volume is about 7% of the total body weight), falling to a trough at 3-4 hours and rising again at 5 hours to decline slowly. Radioactivity was still measurable after 24 hours when it accounted for about 2.5% of the dose (n=3). Calculation of the plasma terminal half-life gave values between 8-10 hours. Biliary levels of radioactivity showed a slow rise to a single peak 4-8 hours after dosing followed by a steady decline, the total amount excreted in 24 hours accounting for 18.3 ± 4.6% (n=6) of the administered dose.

Chromatography of urine (0-24 hour) from all animals indicated the presence of the parent compound (about 1-5%) and large amounts of naphthalene-1,3-diol-positive material (95-99%) which was not detected on the thin-layer chromatograms of urine samples pretreated with β-glucuronidase (Table 2). The liberated aglycone was shown to be a hydroxy-derivative but isomer identification was not possible owing to lack of authentic hydroxy-derivatives for the 3-chloro- and 4-chlorobenzanilides.

Further investigations of the urine from animals dosed with 2-chlorobenzanilide showed that this naphthalene-1,3-diol-positive material was mainly an ether glucuronic acid conjugate (R_f 0.06) of the 4'-hydroxy derivative (R_f 0.51) together with smaller amounts of the 2'-hydroxy derivative (R_f 0.64) (Table 2). Examination of bile showed only the glucuronide (identity not resolved), and methanolic extracts of faeces, which only removed part of the radioactivity, gave the parent compound (R_f 0.61; cyclohexane/acetone 7/3, R_f 0.43). It was assumed that the radioactivity remaining within the faeces (*c.* 50%) was due to tightly bound polymerised phenolic material. More detailed investigations into the metabolism of the compounds were not undertaken.

DISCUSSION

Following oral administration, the majority of the chlorobenzanilides were rapidly excreted from the body, although between 13-17% of the dose was unaccounted for after 3 days. This

TABLE 2

Urinary (0-24 hr) metabolite profile following oral administration of [^{14}C]-chlorobenzanilides to the adult male rat

Dose	unchanged compound	glucuronic acid conjugate		
		Aglycone:		
		2-OH	3-OH	4-OH
2-chlorobenzanilide (9)	3.3 ± 1.6	4.2 ± 1.6	n.d.	92.5 ± 2.1
3-chlorobenzanilide (4)	1.0 ± 0.4	------- 99.0 ± 0.4* -------		
4-chlorobenzanilide (4)	3.1 ± 0.8	------- 96.9 ± 0.8* -------		

Values are expressed as a percentage of the radioactivity excreted in the 0-24 h urine (mean ± 1 s.d.)
The number of animals is denoted in brackets.
* = Identity of the aglycone was not resolved.
n.d. = not detected (less than 0.1% administered dose).

amount corresponds to about 5-8 mg of compound in each animal. From an examination of the excretory profiles, it appears unlikely that much more radioactivity remains to be voided via the urine and that the residual dose is presumably contained within the gastrointestinal tract, as has been shown to be the case with 2-iodobenzanilide /7/.

A slow gut transit time could mean that this phenomenon was due to remnants of unabsorbed dose, but it is more probably conjugated material undergoing enterohepatic cycling. The calculated plasma half-life of 8-10 hours would indicate that up to 88% of the radioactivity would be removed from the plasma within 24 hours. Since only 60% appeared in the urine during that time, there must have been another route of removal. Indeed, a decline in plasma levels at 3-4 hours after administration coincides with a rise to peak values in biliary radioactivity levels, and a second plasma rise is suggestive of reabsorption due to enterohepatic cycling. These observations support the involvement of the biliary route of excretion, a process previously suggested to be of importance for compounds of this nature /7,14/.

It is possible that a portion of the chlorobenzanilides are hydrolysed during their passage through the body and the liberated aniline is further degraded to radioactive carbon dioxide, which was not collected in this study. The cells of *Bacillus sphaericus* and its purified aryl acylamidase have been shown to hydrolyse 2-chlorobenzanilide to liberate aniline /15/, and a *Norcardia* species of soil bacterium has been shown to be able to utilise 2-chlorobenzanilide as its sole source of carbon and nitrogen, degrading the liberated aniline via substituted pyrocatechol, muconic acid, succinate and acetyl CoA /16,17/. However, incubation of monochlorobenzanilides with trypsin, α-chymotrypsin (bovine pancreas) or pepsin (porcine stomach mucosa) produced no detectable aniline /18/ and the specific examination of urine voided from rats dosed with 2-chlorobenzanilide showed no aniline (less than 0.01% dose) /19/. In addition, investigations into the metabolism of [^{14}C]-2-iodobenzanilide in the rat have shown that no (less than 0.2% dose) radioactive carbon dioxide was found in the expired air during the 6 days following dosing /7/.

Glucuronide formation was the major metabolic process for all compounds examined, with the prominent site of hydroxylation on the 2-chloro-isomer being the 4'-position of the aniline ring. Such para-hydroxylation is not unexpected and has been shown to be the predominant pathway of metabolism for many other similar compounds which possess unsubstituted aniline rings /7,8,14,20/.

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