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The metabolism and excretion of [^{14}C] 2- and 4-chlorobenzoic acids in the rat

C.A. Blackledge [a], E.A. Partridge [b], I.D. Wilson [b,*], J.K. Nicholson [c]

[a] *Department of Chemistry, Birkbeck College, Gordon House, 24 Gordon Square, London WC1H 0PP, UK*
[b] *AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*
[c] *Biological Chemistry, Biomedical Sciences Division, Imperial College of Science Technology and Medicine, Sir Alexander Fleming Building, Exhibition Rd, South Kensington, London SW7 2AZ, UK*

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Abstract

The metabolic fate of [^{14}C]-labelled 2 and 4-chlorobenzoic acids (2- and 4-CBA) has been determined in the rat following intraperitoneal (i.p.) administration at 100 mg/kg to male rats. The major route of elimination for both 2- and 4-CBA was urine with $>80\%$ of the dose recovered in the initial 0–24 h after administration. Glycine conjugation was found to be the dominant metabolic fate for both [^{14}C] 2- and 4-CBA however, the position of chloro substitution had a clear effect on the extent of metabolism via this route with *ortho* substitution reducing the extent of metabolism via this pathway.

Keywords: Chlorobenzoic acids; Metabolism; Glycine conjugation; ^{14}C-Labelling

1. Introduction

The metabolic fate of substituted benzoic acids is of continuing interest because of their widespread industrial use and their presence in a wide range of drug molecules. Knowledge of the metabolic fate of these compounds is important in order to devise appropriate methods for determining the exposure of workers handling these materials and also to obtain a more complete understanding of the role of metabolism in their excretion and elimination. During the course of a series of studies on the structure–metabolism relationships within this class of compounds [1–3] we have undertaken an in vivo study of the metabolism of 2- and 4-chlorobenzoic acids (2- and 4-CBAs) in the rat. The metabolism of 2-, 3- and 4-CBA has previously been studied in vivo in the dog [4] and rabbit [5]. In the dog all three CBAs underwent both glycine and glucuronic acid conjugation whilst a proportion of the dose was excreted unchanged (although this was only significant for 2-CBA). In the rabbit the metabolism of 2-, 3- and 4-CBA showed glycine and glucuronic acid conjugation as competing metabolic pathways, although 2-CBA was excreted predomi-

* Corresponding author. Tel.: +44-1625-513424; fax: +44-1625-516962.
E-mail address: ian.wilson@astrazeneca.com (I.D. Wilson)

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nantly as parent compound. More recently the rate of glycine conjugation of 3- and 4-CBA in rat and mouse liver and kidney mitochondria has been investigated [6,7], and the effect of the 4-substituent of benzoic acids on glycine conjugation, including 4-CBA, has been studied in vivo and in vitro [8]. Here the metabolic fate of [^{14}C]-2 and 4-CBA has been studied in the rat following i.p. administration at 100 mg/kg.

2. Experimental

2.1. Chemicals

2-Chlorobenzoic acid [carboxyl-^{14}C] (specific activity 319.5 mCi/mg), purity $>95\%$ was obtained from ICI Cambridge Research Biochemicals Ltd Laboratories and 4-chloro-[ring-U-^{14}C] benzoic acid (specific activity 74.1 mCi/mg), radiochemical purity $>98\%$ from Sigma Radiochemicals. Solvents were of HPLC grade and all other reagents were of analytical grade.

2.2. Dosing

[^{14}C]-Labelled 2-CBA was mixed with unlabelled 2-CBA to give material with a final specific activity of 1.28 μCi/mg, and dissolved in a solution of $NaHCO_3$/NaOH/0.85% physiological saline, 8:22:1 (v/v), to give a final concentration of 83.5 mg/ml. This solution was titrated to pH 7.0 with 11 M HCl for injection. [^{14}C]-labelled 4-CBA was mixed with unlabelled 4-CBA to give material with a final specific activity of 1.34 μCi/mg. This was also dissolved in $NaHCO_3$/NaOH/0.85% physiological saline at a final concentration of 88 mg/ml and titrated to pH 7.0 with 11 M HCl. The specific activities of the dose solutions were calculated from the radioactivity of weighed aliquots of the final dose solutions as measured by liquid scintillation counting (see below).

Male Wistar rats, 220–240 g were housed in individual glass metabolism cages, to allow for the separate collection of urine and faeces, from 24 h before dosing until 120 h post dosing. The room temperature was maintained between 19 and 23°C, relative humidity was 45-70% and the animals were subjected to a controlled lighting regimen of 12 h dark and 12 h artificial light. All the animals had free access to food. Modified R & M No. 1 irradiated diet (Special Diets Services), and fresh water was available ad libitum via water bottles.

Two groups of rats (three males per group) each received a single i.p. dose of either [^{14}C] 2-CBA (100 mg/kg; 128 μCi/kg body weight) or [^{14}C] 4-CBA (100 mg/kg; 134 μCi/kg body weight). Urine, faeces and aqueous cagewash (the water from washing the metabowls) samples were collected for the periods 0–24, 24–48, 48–72, 72–96 and 96–120 h post dose. At the end of the study the rats were killed by inhalation of Fluothane™ ('Halothane', Zeneca Pharmaceuticals) and the carcasses retained. Urine, faeces and carcass were stored at -20°C, and cagewash at $+4$°C, until required for further analysis.

2.3. Sample analysis

2.3.1. Sample preparation

Urine and aqueous cagewash samples were analysed in duplicate by scintillation counting. Aliquots (ca. 100 μl) were weighed into plastic scintillation vials and then mixed with distilled water (900 μl) and Beckman Ready-Value™ Scintillant (10 ml). Carcasses were dissolved in a flask containing a mixture of NaOH (80 g), MeOH (300 ml), distilled water (600 ml) and Triton X-405 (BDH, 300 ml). The flasks were shaken in a waterbath at 50°C for 24 h, and the contents weighed. Four aliquots (ca. 200 μl) were taken from each sample, weighed into plastic scintillation vials and mixed with 4.4 M HNO_3 (100 μl), distilled water (700 μl) and Beckman Ready-Value Scintillant (10 ml). Faecal samples were homogenised with distilled water (1:2 w/v) using a mechanical homogeniser, and the homogenate weight recorded. Four aliquots (approximately 600 mg) were taken from each sample, and combusted using a Packard 306 sample oxidiser. The products of combustion were trapped in Optisorb '1' (9 ml, LKB), to which Optisorb 'S' (12 ml, LKB) was added.

2.3.2. Liquid scintillation counting

All samples were analysed using a Beckman LS 5801, Packard 4640 or equivalent scintillation counter for 10 min, or until 10^4 counts had accumulated. Quench correction was calculated using the 'H' number method (Beckman) or 't SIE' method (Packard).

2.3.3. Thin-layer chromatography

TLC was performed on (a) glass plates precoated with silica gel 60 F254, thickness 0.25 mm (E. Merck), and (b) RP-18 TLC plates F254, thickness 0.25 mm (E. Merck). The solvent systems used for 2-CBA were (A) MeOH–water–TFA, 275:225:1 (v/v) (C_{18} bonded plates) and (B) MeOH–$CHCl_3$–TFA, 225:275:1 (v/v) (silica gel). The solvent systems used for 4-CBA were (A) MeOH–water–TFA, 450:50:1 (v/v) (C_{18} bonded plates) and (B) MeOH–$CHCl_3$–TFA, 35:65:1 (v/v) (silica gel). [^{14}C]-metabolite profiles of urine samples (0–24 and 24–48 h post dose) were obtained by TLC using the solvent systems described above. Urine samples (5–10 μl) were applied to the origin of TLC plates as 2 cm streaks using a Linomat automatic sample applicator (Camag, Switzerland), and the solvent front was allowed to run for 15 cm from the origin. Following chromatography, bands of radioactivity were located using autoradiography and quantified with an Ambis 2-D autoanalyser (Lablogic, Sheffield, UK).

2.4. Isolation of metabolites from urine

Metabolites of 2- and 4-CBA were isolated from 0 to 24-h-post-dose urine samples by solid phase extraction chromatography (SPEC), which was monitored by TLC. Solid phase extraction was carried out using a Mega Bond Elut™ C_{18} bonded silica gel column (Jones Chromatography, Hengoed, UK), which was primed with MeOH (6 ml) and washed with acidified water, pH 2.2 with HCl (6 ml). Urine (10 ml) was acidified to pH 2.2 prior to loading on the column and was then washed with acidified water, (pH 2.2 with HCl) (10 ml). The metabolites were then eluted with a stepwise gradient of increasing eluotropic strength using acidified MeOH–H_2O mixtures as follows: MeOH–H_2O, 1:4 (v/v), MeOH–H_2O, 2:3 (v/v), MeOH–H_2O, 3:2 (v/v), MeOH–H_2O, 4:1 (v/v) and finally MeOH (10 ml). Three aliquots (100 ml) were taken from all eluates and analysed for radioactivity by liquid scintillation counting. The eluates found to contain radioactivity were blown to dryness with nitrogen, freeze-dried, and reconstituted in acidified water, pH 2.2 (2 ml). Samples (5–10 μl) containing radioactive metabolites were applied to the origin of TLC plates as streaks (2 cm), using a Linomat automatic sample applicator, and then run in both solvent systems (A) and (B); as described previously. After drying and autoradiography the plates were analysed on an Ambis 2-D autoanalyser. The above sequence was repeated on individual fractions until the metabolites present were isolated as radiochemically pure compounds, which were reduced to dryness by freeze drying and subsequently as described below.

2.5. Identification of urinary metabolites by mass spectrometry and NMR spectroscopy

Mass spectra were obtained for isolated metabolites directly from the probe of a VG Quattro triple quadrupole mass spectrometer (Micromass Ltd., Altrincham, UK) using fast ion bombardment (FIB) in negative ion mode using a caesium ion source with glycerol as matrix.

^{1}H NMR spectra were obtained using a JEOL GSX 500 NMR spectrometer operating at 11.75 (500 MHz ^{1}H NMR frequency). Spectra were obtained on isolated metabolites, re-dissolved in 600 μl of D_2O, using a dedicated ^{1}H probe operating at ambient temperature (25°C). Typically 128 free induction decays (FIDs) were collected for each sample into 32 768 data points using a spectral width of 6000 Hz and an acquisition time of 2.73 s. A further delay of 2.27 s between pulses was used to ensure that the spectra were fully T_1-relaxed. Residual signals for protons present in the D_2O used to re-dissolve the metabolites were suppressed by applying a gated secondary irradiation (off during acquisition) at the water resonance frequency. An exponential line-broadening factor of 0.2 Hz was applied prior to Fourier transformation. Chemical shifts were referenced

to sodium trimethylsilyl-[2,2,3,3,$^2H_4$]-1-propionate (TSP, 1.0 mg/ml, $\delta = 0.0$).

3. Results

3.1. Excretion and urinary metabolite profiles of 2-CBA

Following dosing with 2-CBA at 100 mg/kg the major route for the excretion of the radiolabel was via the kidney with a total of $74.4 \pm 6.3\%$ of the administered radioactivity recovered in urine for the period 0–120 h post dose (Table 1). A further $8.2 \pm 5.2\%$ of the dose was recovered in faeces and $6.6 \pm 1.8\%$ in the aqueous cagewash for the same period, (the total recovery including carcass was $90.3 \pm 0.9\%$). Excretion was rapid, with $72.2 \pm 6.6\%$ of the dose recovered in urine, and $7.6 \pm 5.3\%$ excreted in the faeces within the first 24 h of administration. RP-TLC analysis, followed by autoradiography revealed two major bands of radioactivity, with R_f values of 0.31 and 0.52 respectively. In some samples the presence of a third, minor, band was detected. The radioactivity at an R_f of 0.31, was tentatively identified as unchanged 2-CBA by co-chromatography with a reference standard.

3.2. Isolation and identification of the major urinary metabolite of 2-CBA

As described in the experimental section SPEC, monitored by liquid scintillation counting and TLC was used to isolate the 2-CBA-related material from urine in order to facilitate identification.

The major metabolite was eluted exclusively in the 40 and 60% MeOH fractions and the putative unchanged parent compound in the 60 and 100% MeOH fraction. The 60% MeOH fraction was therefore subjected to a second SPEC fractionation with MeOH washes of 40, 60, 80 and 100%. The major metabolite was again recovered in the 40% MeOH wash, with unchanged parent eluting in the 80% MeOH fraction. Eluates containing material of similar R_f from both SPEC steps were then combined prior to further analysis.

The identities of the purified materials present in the SPEC separated samples were obtained by MS and NMR spectroscopy. When examined using negative ion FIB–MS the major metabolite was provisionally identified as the glycine conjugate of 2-CBA (i.e. chlorohippuric acid, 2-CBA, $[M–H]^- = 212$ amu). Mass spectrometry of the fraction thought on the basis of TLC to contain unchanged 2-CBA confirmed the tentative identification based on co-chromatography. Further confirmation of the presence of 2-CBA and its glycine conjugate were obtained by ^1H NMR spectroscopy. These spectra were typical of a 2-substituted aromatic ring with, in addition, in the case of the glycine conjugate, the singlet corresponding to the CH_2 resonance of the glycine moiety at $\delta = 4.15$ ppm.

Because of the small quantities of radiolabel present in faeces profiling of faecal radioactivity was not attempted.

3.3. Quantification of 2-CHA and 2-CBA

Quantitative analysis of the 2-CBA and its glycine conjugate present in 0–24-h-post-dose

Table 1
The % recovery of the dose following i.p. administration of 100 mg/kg (2-CBA) to male rats[a]

Time (h)	Urine	Cagewash	Faeces	Carcass	Total
0–24	72.18 ± 6.6	6.05 ± 1.7	7.57 ± 5.3	–	85.80 ± 0.73
24–48	1.67 ± 0.2	0.39 ± 0.1	0.3 ± 0.1	–	2.36 ± 0.23
48–72	0.4 ± 0.04	0.11 ± 0.0	0.12 ± 0.0	–	0.64 ± 0.05
72–96	0.1 ± 0.04	0.04 ± 0.0	0.14 ± 0.1	–	0.28 ± 0.06
96–120	0.07 ± 0.01	0.02 ± 0.0	0.07 ± 0.0	–	0.16 ± 0.01
0–120	74.41 ± 6.3	6.62 ± 1.8	8.21 ± 5.2	1.08 ± 5.2	90.32 ± 0.9

[a] All values quoted are the mean $\pm$ S.E. ($n = 3$).

Table 2
The % recovery of the dose following i.p. administration of 100 mg/kg 4-CBA to male rats[a]

Time (h)	Urine	Cagewash	Faeces	Carcass	Total
0–24	79.71 ± 3.1	3.26 ± 0.7	3.11 ± 0.9	–	86.09 ± 2.27
24–48	0.99 ± 0.5	0.17 ± 0.1	0.38 ± 0.1	–	1.54 ± 0.5
48–72	0.18 ± 0.1	0.05 ± 0.0	0.13 ± 0.0	–	0.35 ± 0.05
72–96	0.07 ± 0.0	0.03 ± 0.0	0.07 ± 0.0	–	0.17 ± 0.0
96–120	0.04 ± 0.0	0.02 ± 0.0	0.05 ± 0.0	–	0.11 ± 0.0
0–120	80.99 ± 3.2	3.53 ± 0.7	3.75 ± 0.7	1.34 ± 0.4	89.60 ± 0.9

[a] All values quoted are the mean ± S.E. ($n = 3$).

urine, was carried out by radio-TLC, using an Ambis 2-D autoanalyser. Roughly equal quantities of 2-cholorobenzoic acid (46.1 ± 0.8%) and 2-chlorohippuric acid (50.1 ± 1.1%) were present with background radioactivity, and minor radioactive bands (2.5 ± 1.5%), accounting for the remainder.

3.4. Excretion and urinary metabolite profiles of 4-CBA

The excretion of radiolabelled material following i.p. dosing of 4-CBA via the urine and faeces, together with aqueous and that remaining in the carcass is summarised in Table 2. The excretion profile of 4-CBA was similar to that obtained for 2-CBA, in that excretion was rapid and predominantly via the urine. In the first 0–24-h period 79.7 ± 3.1% of the administered dose was excreted in the urine, with 3.1 ± 0.9% in the faeces and 3.3 ± 0.7% in the cagewash. The total recovery of dosed radioactive material over the 0–120-h period was 89.6 ± 3.0%, with 81.0 ± 3.2% of the recovered radioactive material being excreted in the urine.

TLC analysis of the 0–24- and 24–48-h-post-dose urine samples revealed the presence of two bands of radioactivity with R_f values of 0.77 and 0.66. The minor [^{14}C]-labelled component, with an R_f of 0.66 co-chromatographed with unchanged 4-chlorobenzoic acid.

3.5. Isolation of the urinary radioactive components of 4-CBA

Similar methods were used to isolate and identify the 4-CBA-related material in 0–24-h-post-dose urine samples to those for 2-CBA as described above. Thus, SPEC, monitored by liquid scintillation counting and TLC, was used to separate the major metabolite of 4-CBA from the minor radioactive component (postulated to be unchanged parent compound) present in the 0–24-h-post-dose urine samples.

The major metabolite eluted predominantly in the 60% MeOH fraction with some further material recovered in the 100% MeOH wash whilst the presumed unchanged parent compound eluted only in the 100% MeOH wash. The repetition of this procedure on the 100% MeOH eluent resulted in the complete separation of the major urinary metabolite of 4-CBA from the putative parent compound. Those eluates containing material with the same R_f were combined prior to further analysis.

3.6. Identification of the major and minor urinary components of 4-CBA

The FIB mass and ^{1}H-NMR spectra of the SPEC-isolated radioactive fractions showed the major urinary metabolite of 4-CBA to be the glycine conjugate of 4-CBA, 4-chlorohippuric acid (4-CHA, $[M–H]^- = 212$ amu). The presence of unchanged parent compound as the minor component eluted ion the 100% MeOH fraction was also confirmed by MS and NMR.

3.7. Quantification of 4-CHA and 4-CBA

Glycine conjugated 4-CBA (4-CHA) was found by TLC to account for 73.8 ± 1.9% of the total

radioactivity detected in the 0–24-h-post-dose urine samples, and unchanged parent compound to account for $8.7 \pm 1.1\%$. Background radioactivity, and unknown radioactive bands ($4.4 \pm 2.2\%$) present on the TLC plates accounted for the remaining radioactivity.

4. Conclusions

These studies clearly show that in the rat the major route of elimination for these chlorobenzoic acids is the urine. For both compounds glycine conjugation was the dominant metabolic fate. In the case of 2-CBA significant excretion unchanged was observed showing that, similar to the results obtained in other species, the position of the chloro-substituent relative to the carboxyl group has an important effect on the metabolic fate of these compounds. This effect on the extent of glycine conjugation for chlorobenzoic acids has been suggested to be a result of steric bulk of the chlorine atom [8]. In general, it has been shown that bulky substituents *ortho* to the carboxylic function of substituted benzoic acids result in a decrease in the extent of glycine conjugation undergone [9]. The minimal effect on glycine conjugation when the chlorine atom is in a position *para* to the carboxyl group supports this hypothesis.

References

[1] F.Y.K. Ghauri, C.A. Blacledge, R.C. Glenn, J.C. Lindon, C.R. Bedell, I.D. Wilson, J.C. Nicholson, Biochem. Pharmacol. 44 (1992) 1935–1946.
[2] B.C. Cupid, C.R. Bedell, J.C. Lindon, I.D. Wilson, J.K. Nicholson, Xenobiotica 26 (1996) 157–176.
[3] B.C. Cupid, E. Holmes, I.D. Wilson, J.C. Lindon, J.K. Nicholson, Xenobiotica 29 (1999) 27–42.
[4] A.J. Quick, J. Biol. Chem. 96 (1932) 83.
[5] H.G. Bray, R.C. Clowes, W.V. Thorpe, K. White, P.B. Wood, Biochem. J. 50 (1952) 583–587.
[6] F. Kasuya, K. Isgarashi, M. Fukui, J. Pharmacobio-Dyn. 13 (1990) 432–440.
[7] F. Kasuya, K. Isarashi, M. Fukui, J. Pharmacobio-Dyn. 14 (1991) 671–677.
[8] T. Nanbo, N. Fukasaku, M. Yamaguchi, H. Takagi, T. Ueda, T. Yokoshima, Chem. Pharm. Bull. 38 (1990) 1753.
[9] A.J. Hutt, J. Caldwell, in: G.J. Mulder (Ed.), Conjugation Reactions in Drug Metabolism, an Integrated Approach, 1990, pp. 273–305.