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A GENERAL METHOD FOR TRITIUM LABELLING OF BENZIMIDAZOLE CARBAMATES BY CATALYTIC EXCHANGE IN DIOXANE SOLUTIONS

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SUMMARY

Benzimidazole carbamates (BZCs) act as inhibitors of the tubulin-microtubule equilibria in eukaryotic organisms. Recently drug resistance to this class of compounds in helminth parasites has been shown to be due to a reduced ability of resistant tubulin to bind BZCs. In order to quantitate the nature of the tubulin-BZC interaction a general method for the specific tritium labelling of BZCs has been developed. The BZCs: mebendazole, oxfendazole, parbendazole, oxibendazole, albendazole and fenbendazole were labelled by catalytic exchange using palladium on calcium carbonate in pure dioxane at 60°C under tritium gas. The position of label incorporation for tritiated albendazole was determined by tritium-NMR as the 4-position of benzimidazole nucleus. The yields for individual BZCs varied from 8 to 68% for a range of specific activity of 0.44 to 13.4 Ci/mmole.

Key-Words: Tritium, catalytic exchange, benzimidazole carbamate, mebendazole, oxfendazole, albendazole.

INTRODUCTION

Drug resistance to the broad-spectrum anthelmintic and antifungal benzimidazole carbamates (BZCs) (Figure 1B, I) has

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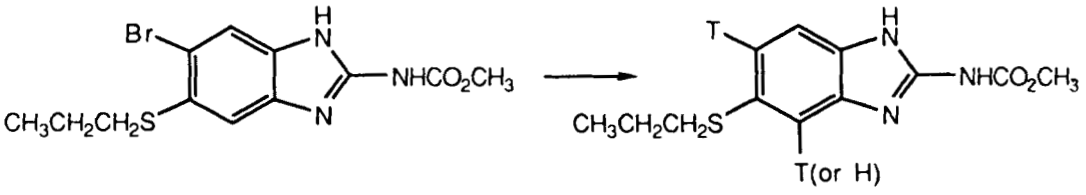
prompted extensive investigations into the biochemical mechanisms underlying the general pharmacodynamics and more specifically mechanisms of resistance of these compounds[1,2]. Investigation of helminth resistance to BZCs has identified changes in the ability of BZCs to bind the structural protein, tubulin, in resistant isolates[3,4]. To enable the accurate quantitation of these phenomena it was essential to develop a method for the synthesis of tritiated BZCs with high specific activity for routine binding studies with tubulin.

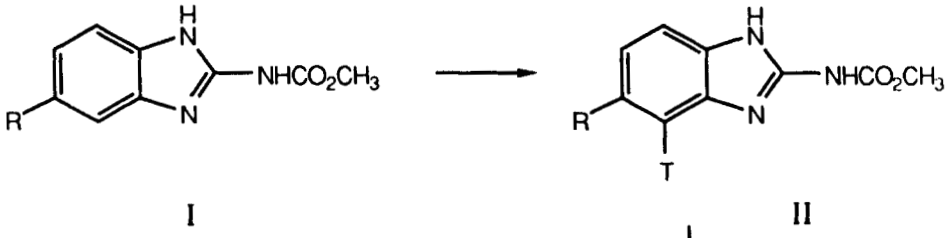
The preparation of specifically labelled [^{3}H]BZCs: mebendazole (MBZ), parbendazole (PBZ) and oxibendazole (OBZ), has been achieved by catalytic dehalogenation of the appropriate bromo-analogues of the parent compounds[5,6]. The synthesis of high specific activity [^{3}H]MBZ requires an extensive six-step synthesis of the 5(6)(4'-bromo) benzoyl analogue and is therefore clearly not suitable for 5(6) non-aryl substituents such as albendazole (ABZ), parbendazole (PBZ) and oxibendazole (OBZ). While a potential synthetic sequence for all BZCs could be accomplished by facile bromination-debromination of 5(6) positions[6], the low specific activity obtained by this route presents considerable difficulty for the quantitation of BZC binding in highly resistant isolates[4].

During preliminary studies of the catalytic dehalogenation of 6(5) bromo ABZ according to the method of Dawson et al.[5] at elevated temperature, it was observed that 1.5 atoms of tritium were incorporated into ABZ (Figure 1(a)). Based on this observation we report the development of a direct tritiation procedure for BZCs by catalytic exchange with palladium on calcium carbonate in dioxane solution, using tritium gas as an isotope source (Figure 1(b)).

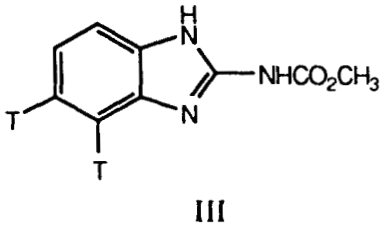
(a)



(b)



for ABZ, OFZ and FBZ



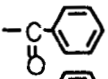
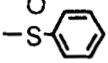
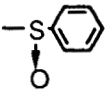
Comp'd	R
MBC	-H
ABZ	-$SCH_2CH_2CH_3$
OBZ	-$OCH_2CH_2CH_3$
PBZ	-$CH_2CH_2CH_2CH_3$
MBZ	
FBZ	
OFZ	

Figure 1. Synthetic route for [^3H] benzimidazole carbamates by (a) [^3H] hydrogenolysis of 6(5) bromo albendazole, and (b) [^3H] hydrogen exchange.

RESULTS AND DISCUSSION

Initial studies of the tritiation of 6(5) Bromo-ABZ at room temperature according to the method of Dawson et al.[5] gave low yield of debromination product (<5%, data not shown).

However heating at 60°C for 30 min gave complete [^{3}H]hydrogenolysis based on the absence of Br-ABZ in HPLC traces of the reaction product. Analysis by mass spectrometry (MS) gave a ratio of molecular ions (M/Z) indicating an isotopic distribution of 2.1: 5.3: 2.6 for ^{1}H: ^{3}H: $^3H_2$ at M/Z 266 (M+1), 268 (M+3) and 270 (M+5) (Figure 3A) as opposed to the expected ^{1}H: ^{3}H distribution previously observed in the preparation of [^{3}H]MBZ from Bromo-MBZ[5]. Assignment of the location of the second site of tritium incorporation as the benzimidazole nucleus was based on the tritium resonances at δ7.15 and δ7.48 (tritium-NMR) which by ghost referencing corresponded to the expected 6-position (achieved by [^{3}H]hydrogenolysis) and the 4-position, respectively (Figure 2B). The latter resonance corresponded to a 33% abundance of tritium in [^{3}H]ABZ. Product distribution based on ^{3}H-NMR and MS analyses gave 21% [^{1}H]ABZ, 7% [4-^{3}H]ABZ, 46% [6-^{3}H]ABZ and 26% [4-^{3}H, 6-^{3}H]ABZ. This suggests that catalytic exchange into the 4 position is highly temperature and time dependent, consideraby slower than the [^{3}H]hydrogenolysis.

Direct tritiation of ABZ by exchange for 45 min at 60°C gave the expected selective incorporation of tritium into the 4-position as judged by ^{3}H-NMR (Figure 2C). MS analysis (Figure 3B) showed only molecular ions M+1 and M+3 present demonstrating that multiple exchange labelling of other aromatic carbons was not significant. Isolated [^{3}H]ABZ possessed an average of 0.55 tritium atoms per molecule.

During the preliminary analysis of the crude reaction, a by-product, methyl benzimidazol-2yl carbamate (MBC) (Figure 1(b), III), was also formed by [^{3}H]hydrogenolysis of the propylthio group. This compound was identified by its

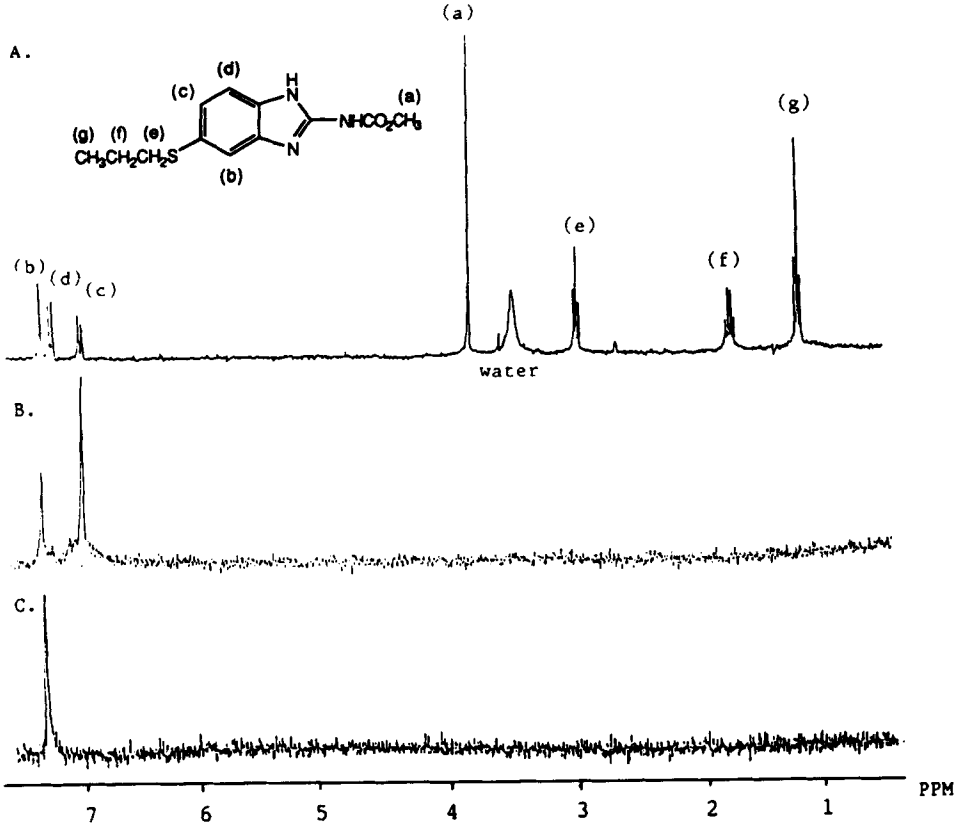


Figure 2. Comparative hydrogen and tritium nuclear magnetic resonance spectra of A. unlabelled albendazole. B. [^{3}H] albendazole derived from 6(5) bromoalbendazole, and C. [^{3}H] albendazole derived from catalytic exchange of albendazole.

co-elution in TLC and HPLC with a pure sample. As expected, based on the [^{3}H]hydrogenolysis results of Br-ABZ, the mixture consisted of ^{1}H, ^{3}H and $^3H_2$ isomers quantitated at 19%, 57% and 24%, respectively by MS. [^{3}H]MBC comprised 30% of the crude product (Table 1).

Exchange labelling of FBZ on a 1mg scale yielded only trace quantities of [^{3}H]FBZ with the major product [^{3}H]MBC isolated in 70% yield. Analogous to the [^{3}H]ABZ synthesis, [^{3}H]MBC demonstrated the $^1H_1$:$^3H_1$:$^3H_2$ ratio consistent with exchange labelling and [^{3}H] hydrogenolysis. [^{3}H]FBZ was

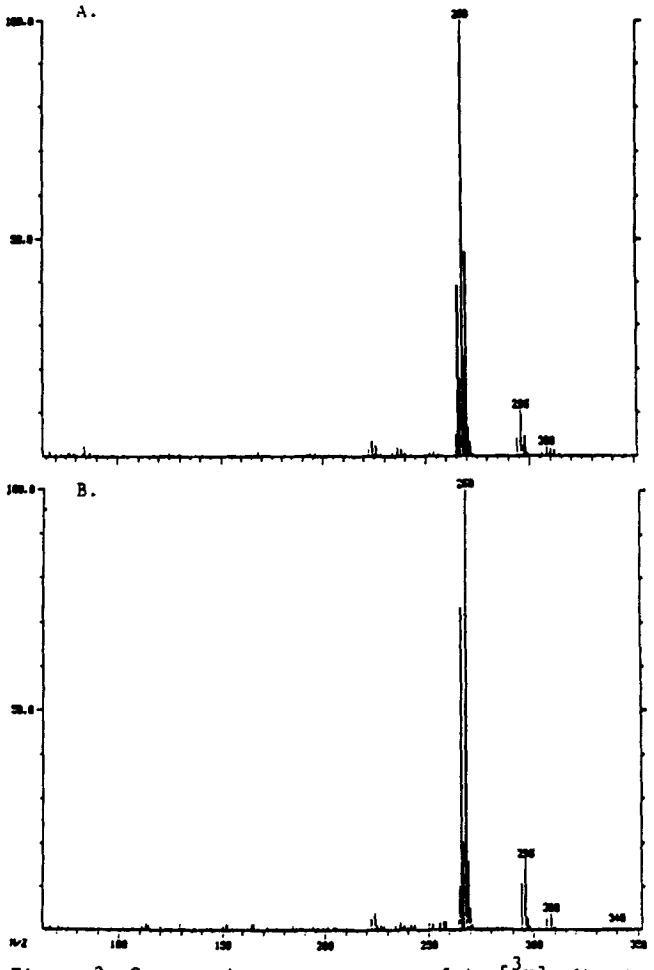


Figure 3. Comparative mass spectra of A. [^{3}H] albendazole derived from 6(5) bromoalbendazole, and B. [^{3}H] albendazole prepared by catalytic exchange. Molecular ion for unlabelled albendazole is M/Z 266.

prepared in albeit low yield (8%) on a 10mg scale reaction, with purification achieved by an initial preparative TLC step in 10% methanol in chloroform prior to HPLC.

Interestingly, tritiation of the phenylsulphinyl derivative, OFZ, was achieved in high yield (56%) with [^{3}H]MBC the major contaminating product. Due to the relatively close HPLC retention times of these compounds on the methanol/ammonium acetate HPLC preparative column,

Table 1. Radiochemical and synthetic data for [^{3}H] labelled benzimidazole carbamates prepared by catalytic exchange

Starting	Product	% Yield	Sp.Act.[a]	HPLC[b] Retention Time	TLC Mobility	Mass Spectra[c]
ABZ	[^{3}H] ABZ[d]	60	4.0	7.35 (29.3)	0.59	266 (100), 268 (42)
	[^{3}H] MBC	30	6.7	3.60 (14.1)	0.55	192 (100), 194 (60), 196 (8)
FBZ	[^{3}H] FBZ[e]	8	0.44	8.40 (34.5)	0.63	300 (100), 302 (<1)
	[^{3}H] MBC[f]	n.d.(70)	n.d.(7.0)	3.60 (14.1)	0.55	192 (100), 194 (85), 196 (12)
MBZ	[^{3}H] MBZ	68	6.0	5.10 (20.1)	0.59	296 (100), 298 (16)
OBZ	[^{3}H] OBZ	30	4.6	6.3 (22.8)	0.57	250 (100), 253 (13)
OFZ	[^{3}H] OFZ	56	9.0	8.10 (-)[g,h]	0.48	316 (100), 318 (48)
	[^{3}H] MBC	18	13.4	3.60 (-)	0.55	192 (80), 194 (100), 196 (30)
PBZ	[^{3}H] PBZ	42	1.0	7.35 (30.9)	0.59	248 (100), 250 (10)

a. Specific activity is quoted in Ci/mmole.
b. HPLC retention times (in minutes) are derived from analytical column using 60% methanol in 0.025M ammonium acetate. Preparative retention times are quoted in brackets for 70% methanol. Flow rates and conditions are as described in the text.
c. Only ^{1}H and ^{3}H molecular ions are shown; abundance is described as percent of base peak (in brackets).
d. Determination of radiochemical purity was achieved by TLC and HPLC. For all labels purity was greater than 98%.
e. [^{3}H] FBZ data obtained from 10mg scale tritiation, for this reaction [^{3}H] MBC yield and sp.act. were not determined.
f. [^{3}H] MBC data derived from 1mg scale, quoted in brackets. [^{3}H] FBZ yield <1%.
g. Analytical retention data was obtained with 32% acetonitrile in 0.025M ammonium acetate as eluant.
h. Preparative HPLC was not undertaken.
n.d., none detected.

purification was carried out by silica column chromatography using the short column vacuum technique[7]. [^{3}H]MBC was eluted in 18% yield (sp.act. 13.4 Ci/mmole) in 2 to 3% methanol in chloroform, with [^{3}H]OFZ eluted in 5% methanol (sp.act. 9.0 Ci/mmole). A residual 9% of the applied radioactivity (TLC Rf 0.59 to 0.76) was not identified. By TLC and HPLC both [^{3}H]MBC and [^{3}H]OFZ were radiochemically pure (>99%) after column chromatography.

[^{3}H]MBZ was prepared in comparable yield and specific activity (68%, 6.0 Ci/mmole) with only minor contamination from the benzhydrol derived by ketone reduction (<10%). This product was readily removed by HPLC. Further characterisation of the alcohol was not undertaken.

[^{3}H]OBZ and [^{3}H]PBZ were prepared in moderate yields (30% and 42%, respectively). However, by TLC both crude reaction products showed the presence of a number of other labelled products constituting greater than 60% of total radioactivity. For [^{3}H]OBZ, silica chromatography identified two contaminating peaks corresponding to TLC R_f 0.74 (46% total radioactivity) and 0.21 (22% total radioactivity). Neither peak could be structurally identified by mass spectrometry; both appeared to contain several ions with M+29 and M+41 adducts suggesting multiple products. [^{3}H]PBZ was eluted from silica chromatography in 4 peaks, with PBZ isolated as 35% of total radioactivity at 2% methanol in chloroform. With the exception of a small peak eluting in 100% chloroform, the residual radioactivity (59%) was isolated in more polar fractions (4 to 5% methanol). Retention data from TLC was not informative as the latter peak gave a broad spread of radioactivity from 0.1 to 0.7 R_f suggesting multiple impurities. This was

confirmed by MS with multiple molecular ions with appropriate adducts identified. This fraction was not further characterised. Both [^{3}H]OBZ and [^{3}H]PBZ fractions of the silica columns were subsequently purified by preparative HPLC.

Although the identification of the position of tritium incorporation has been unequivocally demonstrated for ABZ only, the general applicability of the method for tritiation of other BZCs suggests the involvement of similar exchange reactivity. Confirmation of this hypothesis is currently under investigation with deuterium gas. To our knowledge, exchange labelling into the 4-position of BZs represents a unique reactivity in BZ chemistry which fortuitously is known to be metabolically stable in in vivo systems.

CONCLUSIONS

A general method for the preparation of [^{3}H]BZCs has been achieved by catalytic exchange with Pd on $CaCO_3$ in dioxane under tritium gas at elevated temperature. The applicability of this method has been demonstrated by the preparation of [^{3}H] labelled ABZ, FBZ, MBZ, OBZ, OFZ and PBZ. While the extent of labelling and yields is dependent on the particular compound, the results are compatible with the sensitivity required for biochemical and pharmacokinetic studies. Further, the results confirm the suitability of dioxane as a solvent for exchange labelling.

EXPERIMENTAL

Chemicals

Pure samples of MBZ, OBZ, PBZ, ABZ, OFZ and OBZ were obtained from their respective pharmaceutical

manufacturers[8]. OH-MBZ, OH-MBC and MBC were synthesised as described previously[8].

6-Bromo-ABZ was prepared according to the method for 6-Bromo-OBZ and 6-Bromo-PBZ synthesis[6]. For the tritiation study the crude bromo complex was used, by HPLC this contains 72% Bromo ABZ, the bromine located solely in the 6(5) position.

HPLC purification of tritiated BZCs was carried out using a Waters Associates HPLC system as previously described[6] with a reversed phase C_{18} preparative column (Techsil 5µm, 25cm x 20mm (I.D.), HPLC Technology, United Kingdom). Products were eluted in 70% methanol in 0.025M ammonium acetate. The solvent flow rate was set at 5ml/min and elution times for each system are quoted in Table 2.

Quantitation of yields was achieved using 60% methanol in 0.025M ammonium acetate for ABZ, FBZ, MBZ, OBZ and PBZ and 32% acetonitrile in 0.025M ammonium acetate for OFZ and MBC on a Radpak B (spherical (10µm) C_{18} reverse phase radial compression module (Waters, Sydney Australia) at 1ml/min. 0.5ml of eluant fractions were collected and counted to reconstruct the radioactivity elution profile. The compounds were quantitated by external standardisation with a sample of the appropriate pure BZC.

Chemical ionisation MS was obtained using a Finnegan Quadrupole 3200 Mass Spectrometer interfaced with a 6110 data system with methane as the reagent gas. The fragmentation patterns were compared to pure standards (Table 2). The ratios of ^{1}H and ^{3}H were determined by calculation of the respective areas under the M+1, M+3 and M+5 ion channels for each compound and corrected for ^{13}C and ^{34}S.

Tritium-NMR spectra were obtained on a Bruker CXP-300 Spectrometer at 320MHz with broad band proton decoupling[9]. Chemical shifts were determined by ghost referencing from internal non-tritiated tetramethylsilane in dimethyl sulphoxide-d^6.

Thin layer chromatography of labelled and standard BZCs was determined on Merck Silica H60 aluminium plates using 10% methanol in dichloromethane as the eluant. After development the plates were sectioned in 0.5cm intervals and the radioactivity eluted by shaking in 5ml of Biofluor scintillation cocktail (Dupont, Sydney, Australia) and allowing to stand overnight prior to counting. All liquid scintillation was carried out on a Packard 2000CA Tri-Carb Liquid Scintillation Analyser.

General Procedure for Tritiation

The appropriate BZC (1mg, approx. $3x10^{-6}$ moles) was dissolved in 3ml of dioxane (purified by passage through an alumina column and distilled over sodium and benzophenone) with warming. The solution was mixed with 5% palladium on calcium carbonate (30mg Fluka), evacuated and degassed by freeze-thaw cycles, and stirred under 10Ci of tritium gas (approx. 0.5 atmosphere) at 60°C for 45 min. The catalyst was removed by filtration through a Florosil Sep-pak (Waters) mounted on a 20ml luer lock glass syringe. The reaction vessel was washed with a further 3x3ml of dioxane, then sequentially applied to the Sep-pak. The residual label was eluted with 10ml of dichloromethane. The washings were combined and evaporated under nitrogen at 60°C to dryness. Exchangeable tritium was removed by sequential addition and evaporation of methanol.

Purification and Analysis

(a) Silica column chromatography

The residue reaction product was dissolved in 1% formic acid in chloroform (10ml) and applied under gravity to a vacuum packed dry silica column (30g). On adsorption, the surface was washed with 3x5ml chloroform under vacuum (water pump) and then chloroform (250ml) added in portions to maintain a minimum 1cm solvent reservoir on the column and 20ml fractions collected. The polarity of the eluant was increased in 0.6% methanol increments per 250ml of solvent. The column was terminated after 5% methanol by a 200ml methanol wash. A radioactivity elution profile was constructed from scintillation counts of 50µl samples. Fractions showing good peak homogeneity were pooled and were tentatively identified by elution characteristic of unlabelled compounds in replicate columns and confirmed by TLC and MS. Peaks not consistent with expected products which could not be further identified were not pursued.

(b) High performance liquid chromatography

The residue was dissolved in 20% formic acid in methanol and injected onto the HPLC and the eluted peaks collected and evaporated to dryness under nitrogen to obtain the products.

Purity of each peak was established by dissolution in a 50:50 mixture of methanol and formic acid (1ml) and 1µl samples used for HPLC and TLC analysis. Yields were determined by HPLC quantitation of a 10µl sample of a 1:100 dilution of this solution and by weight of the evaporated solid where appropriate.

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