Synthesis and Stability of Isotopically Labeled *p*-Chloro-*m*-Xylenol (PCMX)

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Received August 12, 1992; accepted April 5, 1993

The synthesis, reaction kinetics, and pH stability of isotopically labeled *p*-chloro-*m*-xylenol (PCMX) were evaluated. While base catalysis was more rapid than acid catalysis, the latter allowed the use of a cosolvent for deuterium and tritium labeling using as little as 250 μL labeled water. Both acid and base catalysis were markedly more rapid than that reported previously for the deuteration of PCMX and related phenols. Isotopic labeling occurred only at the 2 and 6 ring positions, ortho to the phenolic group of PCMX. No deuterium loss was observed after storage for 21 days at 37°C over a pH range of 2–14. Isotopic loss was observed only below pH 2. The prepared ^{3}H-labeled PCMX had a specific activity of 1.18 mCi/mmol, a radiochemical purity of 99.0%, and a chemical purity exceeding 99.0%, with a high stability during prolonged cold storage.

KEY WORDS: *p*-chloro-*m*-xylenol; 4-chloro-3,5-dimethylphenol (PCMX); radiolabel synthesis; isotopes; stability; reaction kinetics; *in vitro* studies.

INTRODUCTION

p-Chloro-*m*-xylenol (4-chloro-3,5-dimethylphenol; PCMX) is the active component of various topical solutions and powders used for the treatment of fungal, yeast, and bacterial infections of the skin (1) and has frequently been substituted for other antiseptic agents used in surgical scrub solutions, including chlorhexidine digluconate and hexachlorophene (2).

While the pharmacokinetics and acute toxicity of this compound have been extensively studied, its potential chronic risk and explicit metabolic pathway have not been fully evaluated, despite reports of liver degeneration and mortality in animal feeding studies, skin irritation in human topical studies, and toxicity to the human fetus (1,3,4).

In order to carry out a series of definitive interspecies *in vitro* metabolism studies using labeled PCMX, it was essential to evaluate the synthesis and stability of the labeled compound. This paper reports the synthesis methodology, kinetics, and extended pH stability of the isotopic labeling of PCMX using deuterium in place of tritium, and the subsequent synthesis of tritium-labeled PCMX for metabolic studies. *In vitro* studies with rat hepatic homogenates using the radiolabeled product indicated that PCMX is hydroxylated to a catechol derivative (5), rather than to a hydroxymethyl derivative (6).

MATERIALS AND METHODS

Reagents

4-Chloro-3,5-dimethylphenol (PCMX; 99.9% pure), deuterium oxide (D_2O), tritiated water, and other chemicals used were obtained from commercial sources. Analysis of the PCMX by ^{1}H-NMR, GC-MS, and HPLC confirmed its structure and showed no impurities.

Isotopic Labeling of PCMX

Deuteration: Base Catalysis. A mixture of 2.0 mmol PCMX (313 mg), 2.0 mL D_2O, and 2.0 mmol NaOD in D_2O (using 40% NaOD in D_2O) was heated in a sealed tube at 100°C for 23 hr, cooled in ice, acidified with 8 drops of 36% DCl in D_2O, and extracted with two 10-mL portions of ether. The combined extracts were dried over anhydrous sodium sulfate and evaporated to give 295 mg (93% recovery) of a pale yellow product, which was confirmed as PCMX-d_2 by GC-MS analysis and NMR observance of loss of the PCMX resonance peak for aromatic protons. Its chemical purity relative to pure PCMX was 100.2% before and 100.0% after purification to a white crystalline solid by passing through a short silica column using 1:2 diethyl ether:petroleum ether. Analysis by C_{18} reversed-phase HPLC and silica gel TLC provided a single peak and a single spot, respectively, with the same retention time and R_f as PCMX. NMR showed 96.6% deuterium incorporation on the aromatic ring (theoretical, 97.5%). Similar results were obtained using 1 mmol NaOD, but using 5 mmol NaOD resulted in markedly reduced deuterium uptake by PCMX. No exchange occurred in D_2O without NaOD.

Alternatively, labeled PCMX was prepared by reacting its sodium salt with D_2O. PCMX-sodium was prepared by adding 11.6 mL of 2.46 *N* NaOH solution (28.5 mmol) in methanol to a solution of 30 mmol (4.70 g) of PCMX in 60 mL of methanol. [Note: 0.95 equiv of NaOH (relative to PCMX) was used since an excess of NaOH (i.e., above 1.00 equiv) was found to inhibit deuterium uptake by PCMX.] PCMX-sodium was isolated by rotary evaporation and vacuum oven drying, and 1.0 mmol (178 mg) was heated with 2.0 mL D_2O in a sealed tube at 100°C for 22 hr, cooled, acidified with 200 μL (3.3 mmol) acetic acid, and extracted with three 10-mL portions 1:1 ether:petroleum ether. The combined extracts were dried over anhydrous sodium sulfate and evaporated to give 152 mg (96% recovery) of a product confirmed as PCMX-d_2 by GC-MS and NMR.

Deuteration: Acid Catalysis. A mixture of 5.58 mmol (874 mg) PCMX, 3.0 mL 1,2-dimethoxyethane (as cosolvent for PCMX, sulfuric acid, and D_2O), 50 μL conc. sulfuric acid, and 250 μL D_2O was stirred and heated at 110°C in a sealed tube for 18.5 hr. After cooling, 6 mL petroleum ether and 3 mL water containing 0.55 g sodium acetate trihydrate were added, the biphasic mixture was shaken, and the organic layer was separated and extracted with 3 mL water. The water extract and the aqueous sodium acetate solution

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0724-8741/93/1000-1466$07.00/0

were each extracted with 3 mL 1:2 ether:petroleum ether. The combined organic extracts were dried over anhydrous sodium sulfate and evaporated to give 858 mg (98% recovery) of a pale yellow product. Its chemical purity was 100.4% before and 100.5% after silica column purification (as described above) to a white crystalline solid, giving a single HPLC peak and TLC spot corresponding to PCMX. NMR analysis indicated that 57.3% deuterium (of the two aromatic proton positions) was incorporated into the aromatic ring (theory: 59.7%).

Kinetics of Base and Acid Catalysis. The kinetics of base catalysis were evaluated over a period of 24 hr at 100°C, using NaOD/PCMX molar ratios of 0.8, 1.0, and 5.0. The kinetics of acid catalysis were examined over the same time and temperature conditions using an H_2SO_4/PCMX mole ratio of 0.17. Percentage deuteration per sampling time was determined from NMR.

Tritiation. Tritium exchange with PCMX aryl protons (ring position 2 or 6) was carried out with the acid-catalyzed cosolvent procedure outlined above, using 250 μL tritiated water (25 mCi) in place of 250 μL D_2O. The ^{3}H-PCMX product had a specific activity (1.18 mCi/mmol) consistent with the target value (ca. 1 mCi/mmol), a radiochemical purity of 99.0%, and a chemical purity exceeding 99%. HPLC analysis using calibrated ^{3}H and UV flow detectors gave a single peak corresponding to pure PCMX.

Apparatus and Procedures

Proton NMR. ^{1}H-NMR spectra were obtained with a Varian T-60A 60-MHz spectrometer, using deuteromethanol as solvent and tetramethylsilane as internal reference. A Varian VXR-500 multinuclear 500-MHz spectrometer was used to obtain high-resolution NMR ^{1}H spectra on samples of PCMX-d2 which had been incubated at 37°C at various pH for prolonged stability testing.

Gas Chromatography–Mass Spectrometry. A Hewlett-Packard 5890 Series II gas chromatograph coupled to a 5971 mass-selective detector, run with G1034B Chemstation software, was used.

HPLC Analysis of Tritiated PCMX. Radiochemical purity of ^{3}H-labeled PCMX was determined on an ALTEX 334 HPLC having Hitachi 100-40 UV and Packard A500 in-line radioactivity detectors and a 150 × 3.9-mm 4-μm Nova Pak C_{18} column run isocratically using 59.6:39.7:0.7 water–methanol–acetic acid at 0.8 mL/min.

RESULTS AND DISCUSSION

Deuteration of PCMX and Its Analytical Confirmation

Ingold *et al.* (7) showed that acidic deuterating agents such as deuterosulfuric acid, and alkaline catalysts in the presence of deuterated water, can deuterate an aromatic ring via ordinary electrophilic substitution and that the types of aromatic substituents present on the ring influence the position(s) of deuterium incorporation (specifically, $-SO_3H$ and $-NO_2$ are meta-directing; $-Cl$, $-Br$, $-CH_3$, $-OCH_3$, $-N(CH_3)_2$, and $-O$ are ortho- and para-directing). These workers found that in the presence of alkali, deuterium uptake by phenol occurred at the ortho and para positions. Hence, the PCMX phenolic oxygen and methyl groups should both activate the two ring positions ortho to the phenolic group (8), enhancing electrophilic substitution of deuterium for hydrogen at these positions.

Consistent with this, ^{1}H-NMR analysis of PCMX reacted for 23 hr at 100°C with D_2O in the presence of NaOD gave resonances only for phenolic proton (phenolic deuterium exchanged during extraction) and the six aliphatic protons (3,5-dimethyl groups) at 4.96 (broad) and 2.28 ppm, respectively. The resonance for the two aromatic protons (6.58 ppm) of PCMX was absent in deuterated PCMX. Dideuteration of the aromatic ring was confirmed by GC-MS analysis.

The mass spectrum of PCMX showed a parent (molecular) ion at *m/e* 156, which was also the base peak. The ratio of the abundances for the PCMX ^{37}Cl/^{35}Cl isotope peaks at *m/e* 158/156 (30–31%) was in reasonable agreement with the natural chlorine isotope ratio (32.4% (9)). Fragmentation loss of the chlorine atom from both PCMX-^{35}Cl and PCMX-^{37}Cl gave the expected ion at *m/e* 121. Other principal PCMX fragments were those at *m/e* 91 [probably due to tropylium ion (10)] and *m/e* 77 (benzene ion).

The mass spectrum of deuterated PCMX showed *m/e* values that were consistently 2 amu units greater than those observed for PCMX [i.e., deuterated PCMX (PCMX): 158 (156), 160 (158), 123 (121), 93 (91), and 79 (77)]. The consistent 2-amu difference (GC-MS) and disappearance of the aromatic proton resonance (NMR) confirmed the product as 2,6-dideutero-PCMX.

Deuteration Mechanism and Kinetics

Ingold *et al.* reported that deuterium exchange in phenol occurred at three ring positions (para and ortho to the phenolic group) when phenol was heated with aqueous aklaki at 100°C for 30–44 days, followed by standing at 25°C for 7–10 days (7). They obtained similar results when phenol was reacted with aqueous alkali in a 0.24–1.00 alkali/phenol molar ratio at 100°C for 96 hr; however, the degree of deuteration was significantly reduced when the alkali/phenol was increased much above unity. These results suggested that the mechanism for deuterium incorporation into phenolic aryl ring locations involved deuterated phenol (Ph-OD) as an electrophilic deuterating agent and phenoxide ion ($Ph\text{-}O^-$) as a nucleophilic aromatic species.

In studies by Tryon *et al.* (11) using acid-catalyzed deuteration of PCMX, other phenols and phenol ethers by deuteroethanol (60°C, 0.001 equivalent of sulfuric acid), a mean incorporation of only 1.5 deuterium atoms into the aromatic ring of PCMX (i.e., 75% of that possible) was observed after a reaction time in excess of 340 hr (>14 days). At 110°C, acid-catalyzed incorporation of 1.8 deuterium atoms into the aromatic ring of *p*-cresol (90% of theoretical) required 200 hr. These results contrast with the full incorporation of three deuterium atoms into the aromatic ring of phenol in a base-catalyzed reaction conducted at 100°C for 96 hr (4 days), reported by Ingold and co-workers (7). From their results with naphthols and naphthol ethers, Tryon *et al.* (11) proposed that β-naphthol ion activates the α-ring position for proton–deuterium exchange, suggesting a requirement for the conservation of aromatic resonance in the electronic configuration of the transition state.

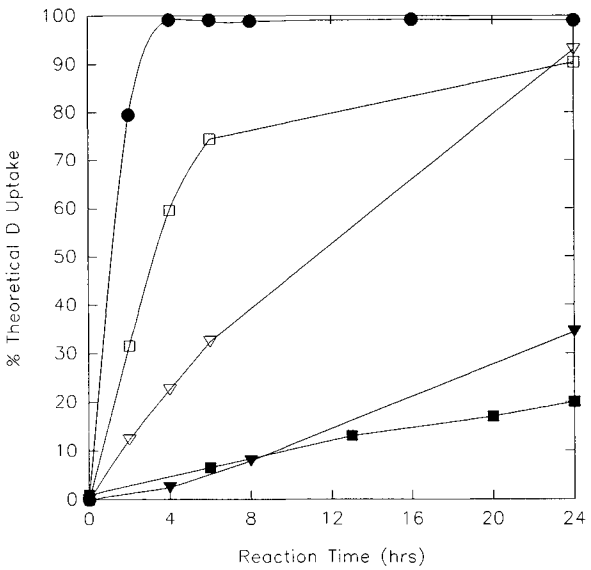

Fig. 1. Rate of deuteration of PCMX using base catalysis at NaOD/PCMX molar ratios of 0.8 (●), 1.0 (▽), and 5.0 (▼) and using acid catalysis (□) (see text for conditions); theoretical D uptake was 59.7% for acid catalysis and 97.5, 97.5, and 98% for base catalysis at NaOD/PCMX molar ratios of 0.8, 1.0, and 5.0, respectively. The rate of deuteration of PCMX using acid catalysis due to Tyron *et al.* (11) is included for comparison (■).

In the present study, the kinetics of base catalysis were evaluated at 100°C and NaOD/PCMX molar ratios of 0.8, 1.0, and 5.0 over a time period of 2–24 hr. For each reaction time evaluated, percentage deuteration was determined from NMR measurements. The results shown in Fig. 1 demonstrate the dramatic effect of excess NaOD in reducing the rate of deuterium incorporation into ring positions in PCMX. Whereas a molar NaOD/PCMX ratio of less than 1.0 resulted in 100% of theoretical uptake of deuterium by PCMX in 4 hr, a molar ratio of 5.0 produced only 2.4% of the theoretical uptake of deuterium in the same reaction time. This is consistent with the hypothesis that PCMX deuterium uptake on the aromatic ring requires the copresence of PCMX phenolate ion and PCMX deuterophenol (i.e., PCMX-OD).

Using acid catalysis at 100°C (H_2SO_4/PCMX mole ratio, 0.17) over a time period of 2–24 hr, we found that the reaction rate was intermediate to that obtained using base-catalyzed 0.8 and 1.0 NaOD/PCMX molar ratios (Figure 1). The relative rates during the early quasi-linear portions of the deuteration curves (Fig. 1) for base catalysis at base equivalents of 0.8, 1.0, and 5.0 and for acid catalysis were 46, 5.5, 1.5, and 14% theoretical D uptake/hr, respectively. The reaction rate obtained for acid-catalyzed PCMX deuteration, while less than base-catalyzed deuteration at 0.8 NaOD/PCMX, was markedly greater than the acid catalyzed results reported by Tryon *et al.* (11) for PCMX deuterium uptake (Fig. 1).

Stability of Isotopically Labeled PCMX

Since the intent of this work was to prepare a radiolabeled PCMX for metabolic studies, it was essential to determine the stability of the isotope label under metabolic conditions at physiological pH and temperature. Aliquots of the same synthetic preparation of PCMX-d_2 were stored at 37°C for 21 days at each buffered pH level from pH 1 to pH 14, including pH 0.1, and water alone. After storage, the PCMX was extracted from the incubation solutions and analyzed by GC-MS and proton NMR.

The GC-MS results (Table I) demonstrated that, not only was the ring isotopic label stable over a prolonged time at physiological pH and temperature, but also it was com-

Table I. pH Stability of PCMX-d2: MS Fragmentation Patterns

	m/e relative abundance											
pH	77	79	91	93	121	123	155	156	157	158	159	160
0.1	16	0	26	0	94	0	3	100	11	32	0	0
1.1	3	4	1	19	18	89	0	19	19	100	32	31
2.0	2	4	0	18	0	91	0	0	23	100	17	30
3.0	1	6	0	20	0	90	0	0	15	100	14	28
4.0	0	5	0	18	1	90	0	0	14	100	11	30
5.0	0	8	0	17	0	93	0	0	15	100	13	29
6.0	0	10	0	23	0	97	0	0	16	100	12	33
7.0	0	7	0	21	1	95	0	0	17	100	14	33
8.0	0	7	0	21	1	95	0	0	18	100	14	29
9.0	0	7	0	20	0	92	0	0	13	100	12	31
10.0	0	8	0	20	0	97	0	0	15	100	14	31
11.0	1	6	0	23	0	100	0	0	15	99	12	30
12.0	0	7	0	22	1	97	0	0	15	100	14	28
13.0	0	4	0	23	0	87	0	0	12	100	11	30
14.0	0	6	0	19	0	91	0	0	13	100	13	28
Mean, 3–14	0	7	0	21	0	94	0	0	15	100	13	30
PCMX	15	0	24	1	92	0	7	100	11	30	0	0
PCMX-d2	0	4	0	20	1	87	0	0	14	100	12	30
d2-2x[a]	2	7	1	16	2	92	0	1	8	100	12	30

[a] PCMX-d2 redeuterated; theoretical %D was 99.94%. ^{1}H-NMR spectrum showed total absence of aromatic proton resonance.

pletely stable at acidic pH levels down to pH 2.0 and at all basic pH levels up to and including pH 14.0. The isotopic label was lost only at extremely low pH (pH 0.1), with only slight losses occurring as low as pH 1.1. The mean values obtained over the pH range from 3 to 14 for the 12 ions studied in the mass spectra of the samples compared well with those obtained for PCMX-d_2 (Table I), confirming the high stability of isotopically-labeled PCMX at physiological pH and temperature. Changes occurring below pH 2.0 and the stability of PCMX-d_2 above pH 2.0 are depicted graphically in Fig. 2 for the parent ions of PCMX and PCMX-d_2 (*m/e* 156 and 158, respectively). The GC-MS results were confirmed via 500-MHz ^{1}H-NMR analysis of selected samples (Table II).

Preparation and Confirmation of Tritium-Labeled PCMX

It was estimated that 5–6 mmol PCMX having a specific activity of ca. 1 mCi/mmol (total activity, 5–6 mCi) would be needed for metabolic studies. To minimize radioactive waste, 250 μL tritiated water having an activity of 25 mCi was used. Base-catalyzed isotopic labeling was considered, but the low volume of (tritiated) water was insufficient to dissolve the desired quantity of PCMX (800–900 mg). Hence, the acid-catalyzed cosolvent method described earlier was used [with 250 μL of tritiated water (25 mCi) in place of 250 μL of D_2O]. Advantages of this method are that a clear, almost colorless homogeneous solution is obtained and that the reaction is facile and requires only simple apparatus.

Estimation of the specific activity of the ^{3}H-PCMX product using both UV measurement against PCMX standards and scintillation counting showed reasonable agreement with the targeted value (ca. 1 mCi/mmol). Quantitative measurement of specific activity and radiochemical purity were determined by HPLC analysis as described above. The radiochemical purity of the ^{3}H-labeled PCMX was determined to be 99.0%. Storage at −20°C as a benzene:ethanol solution for 8 months did not decrease this level of purity. The specific activity determined from the HPLC radioactivity flow detector was 1.18 mCi/mmol. HPLC injection of a sample (14.4 nmol) of the ^{3}H-PCMX product, using tritium and UV (267 nm) monitoring, produced a single radioactivity peak and a single UV peak that had an identical retention time (10.87 min) to that produced on the UV trace from an injection of 15 nmol of unlabeled authentic PCMX.

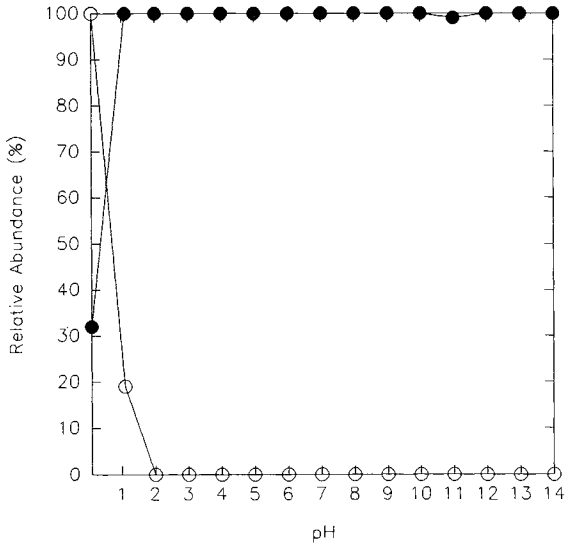

Fig. 2. PCMX-d_2 stability during storage at pH 0.1–14 for 21 days at 37°C. Relative abundance of mass spectral lines at *m/e* 158 (●) and 156 (○), corresponding to the parent ions for PCMX-d_2 and PCMX, respectively.

Table II. pH Stability of PCMX-d2:[a] Aliphatic/Aromatic Peak Area Ratios Determined by High-Field ^{1}H-NMR

pH	Ratio, aliphatic/aromatic[b]	%D[b,c]
14	81.6	96.3
10	58.2	94.8
7	62.2	95.2
3	56.8	94.7
2	41.3	92.7
1.1	10.7	72.0
0.1	3.01[d]	~0[d]
Standards		
PCMX-d_2	87.25	96.4
PCMX	3.01 ± 0.02[e]	~0

[a] Incubated 3 mg PCMX-d_2 in 100 mL aqueous buffer at 37°C for 21 days.
[b] Mean imprecision in the ratio values was of the order of 3–4%, resulting in a mean imprecision in the %D values of the order of 0.3%.
[c] Calculated from %D = [1 − (3 × aromatic/aliphatic ^{1}H integrations)] × 100. Theoretical yield of the PCMX-d_2 used in this study was 97.5%, prepared via base catalysis.
[d] Comparable to that obtained for PCMX standards, indicating complete loss of deuterium from PCMX-d_2.
[e] Standard deviation based on 10 integrations.

Interspecies studies involving *in vitro* hepatic metabolism of PCMX are in progress using labeled PCMX prepared by the methodology reported in this paper. Since the ^{3}H-labeled PCMX contains tritium at either of the two ring positions ortho to the PCMX phenolic group, it has the advantage of distinguishing between aromatic and aliphatic metabolic hydroxylation: the former would result in a 50% decrease in the specific radioactivity of the metabolite and the production of tritiated water; the latter would retain the same specific radioactivity as the parent ^{3}H-PCMX compound. These studies are being carried out to confirm the nature of the principal metabolites of PCMX and to ascertain their ultimate metabolic fate relative to assessing the potential of PCMX for human chronic toxicity.

ACKNOWLEDGMENTS

The authors acknowledge Dr. André D'Avignon (Department of Chemistry, Washington University, St. Louis) for high-field NMR spectra on selected samples of PCMX-d_2 and Dr. William Doub (FDA Division of Drug Analysis, St. Louis) for helpful discussions.

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