Facile synthesis of high specific activity 4-[1-^{14}C]butyl-1,2-diphenylpyrazolidine-3,5-dione (phenylbutazone) using nucleophilic substitution

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Abstract

Metabolism, environmental fate, and low concentration food residue studies would be facilitated by the use of radiolabeled test articles which can be readily quantified within complex matrices. However, radiochemical approaches for such studies require high specific activities to allow analytical detection of correspondingly low masses of test article. The synthesis of high specific activity (>50 μCi/μmol) [^{14}C] radiolabeled phenylbutazone presents a challenge using existing methodology, mainly due to the low solvent volumes required and vigorous refluxing needed to close the pyrazolidinedione ring. Herein we report on the significant modification of an existing method that allows the synthesis of low masses of high specific activity (>50 μCi/μmol) [^{14}C]-phenylbutazone under mild conditions with simple purification and high yield. The closure of the pyrazolidinedione ring of 1,2 diphenyl-3,5-pyrazolidinedione was accomplished as a first step with unlabeled 1,2-diphenylhydrazine and diethyl malonate in 32% yield under gram-scale conditions, which avoided the challenges of low solvent use and vigorous refluxing. Low mass of high specific activity n-[1-^{14}C]-butyl bromide was then added via a nucleophilic substitution reaction as a final step. Yields ranged from 65-92% during multiple synthetic attempts with unlabeled butyl bromide, and were greatly influenced by reaction stoichiometry and the selection of base.

Keywords:

Phenylbutazone, anti-inflammatory, [^{14}C]-radiolabel, butyl bromide, pyrazolidinedione, nucleophilic substitution, stoichiometry, NSAID

1. Introduction

Phenylbutazone (PB; 4-butyl-1,2-diphenylpyrazolidine-3,5-dione) is a nonsteroidal anti-inflammatory (NSAID) drug. It is widely used in animals, especially horses, to provide relief from acute and chronic inflammatory pain.[1,2] Because of concerns of related to food safety, PB is not allowed for use in food animals. Investigations on the partitioning of PB within biological fluids, its metabolism in animals, and laboratory-scale environmental fate and transport are supported with high specific activity, radioisotopically-labeled material. Subsequent use of liquid scintillation counting, tissue combustion analysis, or radiochemical detection during chromatography are commonly used to quantitatively and qualitatively assess chemical fate and disposition, but such experiments are facilitated by high specific activity test articles, especially when low masses of analyte are encountered (as in tissue residue experiments). PB is typically produced by a condensation reaction between *n*-butyl-diethylmalonate and 1,2-diphenylhydrazine in the presence of bases such as sodium ethoxide, potassium *t*-butoxide, or sodium hydride in 40-65% yield at high temperatures.[3-5] This reaction is performed on a reasonably large (milligram or gram) scale. The economical synthesis of high specific activity, radiolabeled PB requires microgram scale reactions using limited solvent, while limiting the consumption of expensive radiolabeled reagent.

To the best of our knowledge there is no literature that describes a methodology for synthesizing high specific activity [^{14}C]-phenylbutazone (>50 μCi/μmol) at the microgram scale. Ayrey and Yeomans[5] demonstrated the synthesis of low specific activity PB (~0.3 μCi/μmol) using a reasonably high mass of reagents (e.g. high milligram scale). The synthesis was accomplished in one-pot with a multi-step temperature gradient via the condensation of [^{14}C]-diethyl malonate (1:20 ratio of radiochemical:unlabeled compound), butyl bromide, and 1,2-diphenylhydrazine in the presence of sodium ethoxide (Fig. 1). The

reaction required a stoichiometric amount of moisture-sensitive base, that suffered from highly variable yields if any residual water from solvents, reagents, or glassware were present; a highly detrimental condition when attempting micromolar scale synthesis of a high specific activity product. We were not able to adapt the method of Ayrey and Yeomans[5] to the micromolar scale to accomplish the synthesis of high specific activity radiolabeled PB since direct scale-down required low solvent volume (<5 μL), ultra-dry conditions, three stages of vigorous heating, as well as a tightly controlled stoichiometric amount of sodium ethoxide base. Therefore, there was a need to discover a robust, reproducible, and high-yielding reaction to make radiolabeled PB which required low quantities of solvent and radiolabeled reagents. Herein a simple, robust, reproducible, and high-yielding methodology to synthesize radiolabeled PB is described.

2. Result and Discussion

The initial reaction attempt was conducted at the millimolar scale with unlabeled reagents according to the published method of Ayrey and Yeomans.[5] An equimolar (4.74 mmol) condensation of diethyl malonate, butyl bromide, and 1,2-diphenylhydrazine was performed in the presence of sodium ethoxide and absolute ethanol with stepwise heating to 150 ^{o}C.[5] The reaction had very acceptable yields (71%) at the millimolar scale. However, for the high-specific activity radiolabeled synthesis of PB, the original goal was to perform the reaction with ~5 μmol (800 μg; 250 μCi) of radiolabeled diethyl malonate. Thus, a series of reactions that varied the amount of diethyl malonate, ranging from 4.74 mmol to 0.15 mmol, was performed to investigate suitability of the reaction at these lower scales (data not shown). Phenylbutazone product was observed in reactions that used diethyl malonate at amounts greater than 0.59 mmol, but product was not observed when diethyl malonate amounts were below 0.3 mmol. Yields were 30-50% when diethyl malonate was greater than

0.59 mmol as assessed by gas chromatography-mass spectrometry (GC-MS). Further reactions were conducted at 0.15 mmol diethyl malonate with different solvents (toluene, chlorobenzene, and xylene) to investigate solvent influences on the condensation reaction; however, no product was detected by GC-MS. Condensation reactions between pre-alkylated butyl diethyl malonate (0.1 mmol) and 1,2-diphenylhydrazine in the presence of sodium ethoxide or potassium *tert*-butoxide bases were also unsuccessful.[6] Thus, another approach was developed to make high specific activity [^{14}C]-PB at a small scale with reasonably high chemical yield. One element of the new approach involved introducing the radiolabel at the final synthetic step rather than during an initial reaction step. As a consequence, radiolabeled diethyl malonate was replaced with radiolabeled butyl bromide (Fig. 2). An additional element was maintaining an optimized stoichiometry for all reagents in the final reaction step. Finally, use of a base, i.e. potassium carbonate, that could be gravimetrically measured with high accuracy replaced sodium ethoxide, mentioned in the published literature, but which suffered from variable base strength.

Since results from the initial millimolar scale reactions indicated that cyclization was the rate-limiting step, gram-scale synthesis of 1,2- diphenyl-3,5-pyrazolidinedione (**3**) was performed first, and resulted in the facile production of desired product (32%). Thus, sufficient amounts of a rate-limiting intermediate (**3**) were available to facilitate numerous unlabeled reactions at the scale needed for radiolabeled butyl bromide introduction via nucleophilic substitution (Fig. 2). 1,2 diphenyl-3,5-pyrazolidinedione (**3**) was synthesized via condensation of 1,2-diphenylhydrazine (**1**) and diethyl malonate (**2**) in the presence of sodium hydride base in chlorobenzene.[7] Improvements to the published method[5] included introduction of sodium hydride base, a solid which resulted in high confidence in the base stoichiometry, and the use of chlorobenzene as solvent, which was less hygroscopic than ethanol, and which possessed a higher boiling point, facilitating the first step of ring closure.

Alkylation of 2.4 equivalents of **3** with 1 equivalent of butyl bromide (**4;** 5 μmol; 685 μg; 0.537 μL) in the presence of 2 equivalents of potassium carbonate base and a catalytic amount of sodium iodide yielded 60% (**5**) by GC-MS (Reaction 1, Table 1), and 30% dibutylated by-product. Incomplete and variable formation of sodium ethoxide from sodium metal and absolute ethanol was detrimental, and contributed to lower yields of (**5**). Solid potassium carbonate was a more suitable base than liquid sodium ethoxide used by Ayrey and Yeomans,[5] and the higher molecular weight and lower hygroscopic properties made it preferable over sodium hydride in this low mass synthetic step. Subsequently, the monoalkylation reaction was optimized by varying the equivalents of K_2CO_3 and (**3**) (Table 1). The scale for optimizing the reaction was chosen on the basis of availability of radiolabel butyl bromide (5 μmol, ~250 μCi). Acetonitrile was a preferred solvent for the nucleophilic substitution reaction because of its polar character and ability to be dried adequately. However, the reaction was also run with hexane since the radiolabeled butyl bromide was packaged in hexane. During nitrogen flushing at 0°C biphasic conditions occurred, but a single phase existed when heating to 55°C during the reaction. Knovenagel condensation with 1,2-diphenyl-3,5-pyrazolidinedione (**3**) and butanal followed by hydrogenation could also have produced (**5**).[8] However, this approach was not followed since it required two additional steps with putative losses, obviously undesirable once the radiolabel had been introduced.

As shown in Table 1, use of 2.4 equiv of **3** resulted in good conversion but with more dibutylation, whereas the use of 6 equiv of **3** slowed the reaction and resulted in low yield at 24 h. The optimized reaction 3 conditions (Table 1) produced the highest yield of **5**, and were repeated seven times with unlabeled reagents with yields that ranged from 65-92% of PB (**5**) by GC-MS. Pure **5** was produced for NMR analysis by purification with silica gel thin layer chromatography (TLC). Optimized reaction 3 conditions were applied to radiolabeled PB

synthesis utilizing n-[1-^{14}C]-butyl bromide, which was directly used from the ampule in which it was shipped (250 μCi in 1 ml hexane). No attempt to evaporate the hexane (BP 68.5°C) was pursued due to the reasonably volatile nature of butyl bromide (BP 101.4°C). After the extraction of the radiolabeled reaction with dichloromethane, the organic layer was dried over sodium sulfate, and crude radiochemical (83.7 μCi) was purified by prep TLC developed in 1:1 hexane:ether. Radiolabeled **5** was obtained in 9% yield (Rf = 0.3; 23 μCi; >98% pure; 57 μCi/μmol), which was confirmed by GC-MS. A significant amount of dibutylation unexpectedly occurred as the main by-product (21.7 μCi; Rf = 0.6, 9% yield; 4,4-dibutyl-1,2-diphenylpyrazolidine-3,5-dione). An uncharacterized oxidation product [5,9,10] was also observed near the origin (Rf = 0.04; 23.3 μCi, 9%). The aqueous layer contained most of the remaining radioactivity, i.e. 166 μCi. The radiolabeled synthesis results were negatively affected by unexpectedly high radiochemical content (656 μCi; 13 μmol) of n-[1-^{14}C]-butyl bromide. The stoichiometric excess (2.6-fold) of butyl bromide (13 vs 5 μmol) in the commercial radiolabeled product caused a shift to dialkylation rather than the targeted monobutylation. According to the reactions in Table 1, a shift from the optimal ratio of 4:1 (**3** to butyl bromide) to this new ratio of 1.5:1 would favor dibutylation. For example, lowering the optimal ratio to 2.4:1, as in reaction 1 (Table 1), resulted in a significant increase in dibutylated by-product (15% vs. 30% for reaction 3). The lack of full mass balance of radioactivity was probably due to the semi-volatile nature of n-[1-^{14}C]-butyl bromide (BP ~100°C), which could be lost during reaction heating (55°C) or rotary evaporation of organic extracts (45°C under vacuum). Nevertheless, the concept of synthesizing a low mass phenylbutazone with high [^{14}C] specific activity needed to conduct future food fate studies in our laboratory was proven.

3. Conclusion

A simple, mild, robust, relatively high-yielding method to prepare radiolabeled phenylbutazone was developed. It is recommended that the exact stoichiometry of all reagents developed in these studies is used for highest yield, including K_2CO_3 base, but especially butyl bromide so that it will not be evaporated under the positive nitrogen pressure maintained in this reaction, or result in the formation of the undesirable dibutylated product.

4. Experimental

Diethylmalonate, butyl bromide, 1,2-diphenylhydrazine were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Acetonitrile was distilled over CaH_2 and stored over 4Å molecular sieves (Linde). Radiolabeled n-[1-^{14}C]-butyl bromide was purchased from American Radiolabeled Chemicals (57 μCi/μmol; 250 μCi; St. Louis, MO). A radioactive assay of the small amount of n-[1-^{14}C]-butyl bromide remaining in the shipped ampoule after its use in the reaction yielded a considerably higher amount of actual material received, i.e. 656 μCi, 13 μmol; 1.8 mg, which was 2.6-fold more than stated. NMR spectra were obtained on a 400 MHz Bruker BZH400/89 spectrometer. For GC-MS, a Hewlett Packard 5890 with auto sampler (75-300 ^{o}C, 5 min solvent delay, temperature gradient 10 ^{o}/min, final hold 10 min) was used. Prep TLC purification of PB was performed using silica gel GF plate (Analtech, Newark, DE; 250 μm, 5 x 20 cm), and was visualized with ultraviolet light (280 nm; for non-radiolabeled detection) or a System 2000 Imaging Scanner (Bioscan, Washington, DC; radiochemical detection).

Synthesis of 1, 2-diphenyl-3, 5-pyrazolidinedione (3):

1,2-Diphenyl-3,5-pyrazolidinedione **(3)** was synthesized according to an published method.[3b] To a mixture of 1,2-diphenylhydrazine (**1**; 1.84 g, 10 mmol), diethyl malonate (**2;** 1.6 g, 10 mmol) and chlorobenzene (8 mL), sodium hydride (60% mineral oil; 480 mg, 12 mmol) was added in three portions at 0 ^{o}C. Reaction was refluxed for 5 h then cooled to room

temperature. Water (20 mL) was added to the flask to quench the reaction. The reaction was extracted with dichloromethane (4 x 20 mL). The aqueous layer was acidified with 2 M HCl to pH 2 and then extracted with dichloromethane (3 x 20 mL). Combined organic layers were dried over sodium sulfate and concentrated in a rotary evaporator to obtain 1.2 g of crude 1,2-diphenyl-3,5-pyrazolidinedione (**3**) in 32 % yield (0.8 g, 3.2 mmol). Sample was recrystallized from absolute ethanol. Product was confirmed by ^{1}H-, ^{13}C-NMR and GC-MS. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 3.57 (s, 2H), 7.18-7.37 (m, 10H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 38.7, 124.9, 129.3, 131.3, 137.5, 168.7. GC-MS (m/z, relative intensity) 252 (M+, 50), 183 (58), 105 (21), 77 (100).

Synthesis of non-radiolabeled phenylbutazone (5):

To a 10 mL vial, 1,2-diphenyl-3,5-pyrazolidinedione (**3**, 50 mg, 200 μmol, 4 equiv), potassium carbonate (10 mg, 75 μmol, 1.5 equiv), sodium iodide (single crystal), butyl bromide (**4**, 7 mg, 5.5 μL, 50 μmol, 1 equiv), acetonitrile (5 mL) and hexane (2.5 mL) were added. The reaction vial was placed into an ice bath while nitrogen was bubbled into the vial for 15 minutes with an exit needle to exclude oxygen. Then the exit needle was removed and the nitrogen inlet was replaced by N_2 balloon and sealed to the vial with Parafilm®. The reaction was removed from the ice bath and heated at 55 ºC for 22 h, after which time the reaction was cooled to room temperature. The solvent was removed via rotary evaporation. Water (5 mL) was added to the residue and extracted with dichloromethane (4 mL x 3). Combined organic layers were dried over sodium sulfate and the organic layer was concentrated by a stream of nitrogen, leaving crude PB (**5**), which was purified with TLC developed with 1:1 hexane:ether (12 mg, 38.9 μmol; 78% yield). ^{1}H- and ^{13}C-NMR[11], and GC-MS[12] matched with literature values. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.4-7.1 (m, 10H), 3.40 (t, *J*=8 Hz, 1H), 2.12-2.10 (m, 2H), 1.53-1.43 (m, 2H), 1.42-1.31(m, 2H), 0.90 (t, *J* = 8

Hz, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 170.6, 135.9, 127.0, 122.7, 46.5, 28.1, 24.4, 22.3, 13.9. GC-MS (m/z, relative intensity) 308 (M+, 35), 252 (9), 183 (80), 105 (16), 77 (100).

Synthesis of [^{14}C]radiolabelled phenylbutazone (5):

To an ice-cold 4 mL vial 1,2-diphenyl-3,5-pyrazolidinedione (**3**, 4.4 mg, 17.4 μmol, 4 equiv), potassium carbonate (1.2 mg, 8.7 μmol, 2 equiv), sodium iodide (single crystal), n-[1-^{14}C]-butyl bromide in 0.25 mL hexane (assumed 596 mg, 4.35 μmol, 1 equiv, ~250 μCi), acetonitrile (0.5 mL) were added. Reaction was flushed with nitrogen for 15 minutes over icebath. The reaction was heated to 55 ^{o}C for 23 h, and allowed to cool to room temperature. Water (5 mL) was added to quench the reaction and then the contents were extracted with dichloromethane (3 mL x 4). Combined organic layers were dried over sodium sulfate, and organic layer was concentrated by a stream of nitrogen. Radiolabeled phenylbutazone was purified by TLC with 1:1 hexane:ether development. Characterization of radiolabeled phenylbutazone was confirmed by GC-MS. GC-MS (m/z, relative intensity) 310 (M+, 27), 252 (8), 183 (84), 105 (16), 77 (100).

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Table 1. Optimization of reaction conditions for the final step in the synthesis of [^{12}C]phenylbutazone (**5**) utilizing 1, 2-diphenyl-3, 5-pyrazolidinedione (**3**), potassium carbonate base, and butyl bromide. Reactions were conducted with 1 equivalent of [^{12}C]butyl bromide (5 μmol) in 0.5 mL acetonitrile and 0.25 mL hexane.

Reaction #	Equiv. K_2CO_3	Equiv. **3**	Time (h)	% (**5**)	% Dibutylation
1	2	2.4	22	60	30
2	2	6	24	3	1
3	2	4	18	85 [a]	15 [a]
4	2	4	6	20	0
5	1.5	4	18	66	4

[a] Value is the mean of seven reactions.

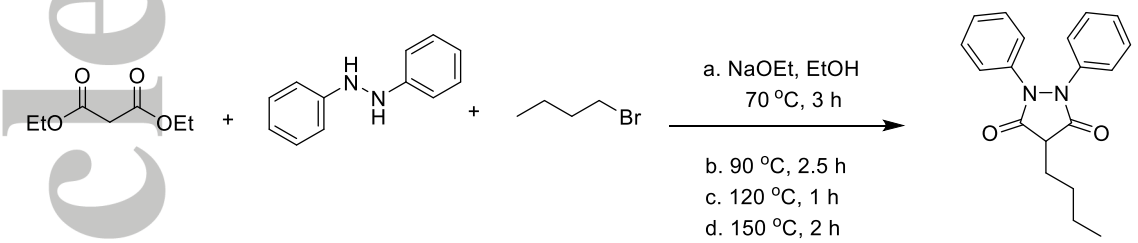

Figure 1. Synthesis of [^{14}C]-radiolabeled phenylbutazone by the one step method of Ayrey and Yeomans (1974). The method involved initial condensation of all three reagents at 70-90°C, solvent evaporation and pyrazolidinedione ring closure at 120-150°C to yield phenylbutazone.

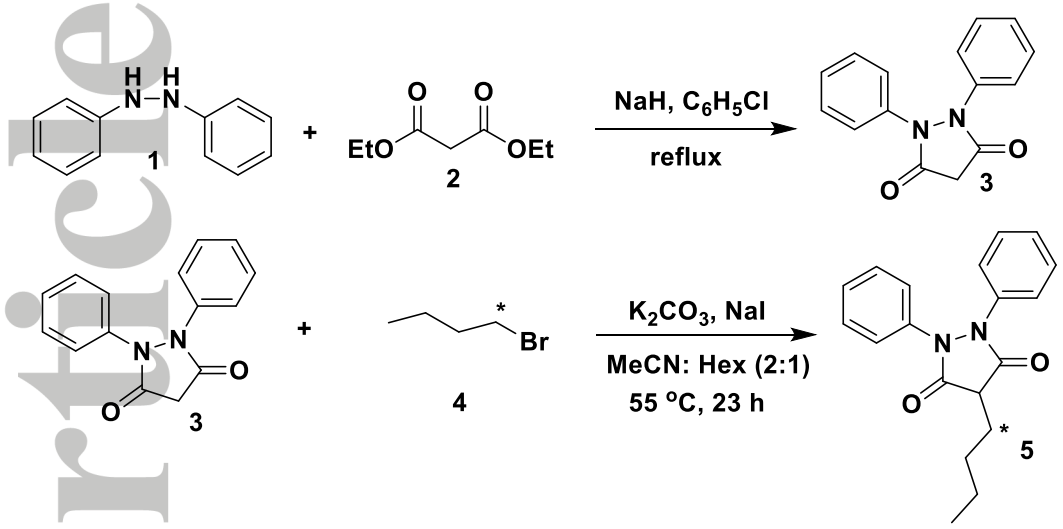

Figure 2: Synthesis approach for [^{14}C]-phenylbutazone (**5**); * indicates position of [^{14}C] labeled carbon. The first step involves formation of the unlabeled pyrazolidinedione ring (**3**) by condensation of 1,2-diphenylhydrazine (**1**) and diethyl malonate (**2**). The introduction of the radiolabel in the form of [^{14}C]-butyl bromide (**4**) is performed as the final step in a nucleophilic substitution reaction.