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SYNTHESIS AND ANALYSIS OF [4 -^{14}C] PHENYLBUTAZONE

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[4-^{14}C] phenylbutazone (4-butyl-1,2-diphenyl-3,5-[4-^{14}C] pyrazolidinedione) has been synthesised on the 5 mmole scale. [2-^{14}C] Diethyl malonate was reacted with n-butyl bromide and the product condensed with hydrazobenzene in the same reaction vessel. The radiochemical yield of highly purified product was 48%. It is proposed that care should be taken in analyses for metabolic products, particularly by TLC, since 4-hydroxyphenylbutazone was shown to be an oxidation product of phenylbutazone in solution and on TLC plates under quite mild conditions. A pure sample of 4-hydroxy[4-^{14}C] phenylbutazone was isolated.

Introduction

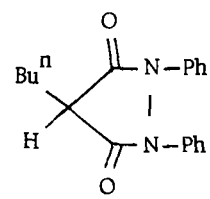
Phenylbutazone is a widely used drug for the treatment of inflammatory and rheumatic conditions in humans and animals but its metabolic fate is only partly explored.[1] The drug has also been misused in the treatment of racehorses. Metabolism studies are most easily carried out with radioisotopically labelled material and a ^{14}C ring labelled phenylbutazone was required for use in such studies. The commercial availability of [2-^{14}C] diethyl malonate allowed synthesis of [4-^{14}C] phenylbutazone. In the course of this work we discovered that 4-hydroxyphenylbutazone a likely metabolic product is formed rather easily under a variety of conditions.

Synthesis of [4 - ^{14}C] phenylbutazone

The synthetic route chosen was that described in patent literature:[2]

$$Bu^n\,\dot{B}r + CH_2\,.\,[CO_2Et]_2 \xrightarrow[EtOH]{NaOEt} Bu^n\,.\,CH\,.\,[CO_2Et]_2 \qquad (1)$$

$$Bu^n\,.\,CH\,.\,[CO_2Et]_2 + [Ph\,.\,NH]_2 \xrightarrow{NaOEt} \qquad (2)$$



All the reactants were placed together in the reaction vessel and conditions controlled initially for the formation of n-butyl diethyl malonate [Reaction (1)]. Subsequently conditions were altered to effect ring closure [Reaction (2)].

Experimental procedures described previously did not always give the yields claimed by their originators and had to be improved and modified for small-scale preparation. Repeated trial syntheses with inactive materials showed that there was no advantage in carrying out the synthesis in two completely separate stages. On the contrary the use of only one reaction vessel with subsequent reduced manipulation led to improved yields and was preferred for the radiochemical synthesis. Efficient stirring of the reaction mixture was found to be essential and this was accomplished by use of a "Spinfin" magnetic stirrer.[3] The amount of sodium ethoxide present was found to be critical and was carefully regulated by dropwise addition from a hypodermic syringe of the calculated volume of a standard solution. High yields were obtained only when anhydrous conditions were maintained.

Isolation and purification of the product were effected by procedures more suitable for a small quantity of radioactive material than the original methods. The product, initially in the form of its sodium salt, was extracted into aqueous solution. Extraction with chloroform removed unreacted hydrazobenzene and several by-products. Acidification of the aqueous solution, followed by extraction of the precipitated material into chloroform and evaporation of the solvent yielded crude product. Chromatography on a column of silica gel then gave a high degree of purification. In trial syntheses yields varied from 55 to 61%. In the final radiochemical synthesis, the chemical yield, calculated on the weight of product from the column was 71%, the radiochemical yield 61% and the radiochemical purity 95%. Dilution with pure inactive phenylbutazone followed by recrystallization from ether gave a product whose purity was established to be 98 $\pm$ 2% by reverse isotope dilution analysis. The radiochemical yield of this pure product was 48%. Thin-layer chromatography was shown to be an unsatisfactory method for analysis of phenylbutazone (see below) but was used as a confirmatory test of radiochemical purity.

Oxidation of phenylbutazone in organic solvents

Thin layer chromatography (TLC) of pure phenylbutazone with various organic solvents gave two separate spots. The minor component varied in abundance but could never be eliminated. Two dimensional chromatography revealed that the same minor component separated on the second development despite the fact that it had already apparently been removed during the first development. In the second development the two "impurity" spots had identical R_f values. Thus some degradative reaction, most probably oxidation of phenylbutazone was suspected.

Phenylbutazone has previously been shown to undergo oxidation by atmospheric oxygen.[4] Oxidation also occurs in alkaline solution.[5] In both cases the product was

shown to be 4-hydroxyphenylbutazone. Thus the minor component observed on the thin-layer chromatogram was strongly suspected to be 4-hydroxyphenylbutazone, and it was thought likely that this product could be formed in solutions of phenylbutazone in organic solvents. This was confirmed by heating an acetone solution of phenylbutazone in air and then separating the oxidation product from phenylbutazone by extracting with sodium carbonate solution. The isolated material was purified and identified as 4-hydroxyphenylbutazone by its melting point, microanalysis, mass, infra-red and proton magnetic resonance spectra. It had the same R_f values as the impurity spot of phenylbutazone.

The mass spectrum indicated the required molecular weight of 324. A strong band at 3370 cm^{-1} in the infrared spectrum indicated the presence of a hydroxyl group. The p. m. r. spectrum was measured in deutero chloroform and compared with that of phenylbutazone. The spectrum of phenylbutazone contained a triplet at 6.73 τ due to the single hydrogen atom attached to carbon atom 4 of the ring. In the spectrum of the oxidation product this triplet was absent but a new singlet at 6.1 τ indicated the presence of a hydroxyl proton. Equilibration with deuterated water caused the singlet to disappear confirming that it was due to a hydroxyl proton.

Further qualitative experiments showed that 4-hydroxyphenylbutazone was also formed in varying amounts in chloroform, ethanol, ether and hexane at room temperature and at 0 °C. TLC estimates of yield indicated that phenylbutazone was most stable in ether in the dark at 0 °C and least stable in acetone. Oxidation was accelerated by increase in temperature and also by visible light and exposure to ultraviolet light. Thus, we recommend that in any metabolic study involving labelled phenylbutazone great care should be exercised if 4-hydroxyphenylbutazone is suspected to be a metabolic product.

4-hydroxy-[4-^{14}C] phenylbutazone

TLC examination of the later fractions from column chromatography of the labelled product showed the presence of 4-hydroxy[4-^{14}C]phenylbutazone. This was probably formed during both the synthesis and the isolation procedure. TLC analysis followed by radioscanning showed the proportion of 4-hydroxy[4-^{14}C]phenylbutazone in fraction 13 to be approximately 20%, representing about 4% of the total starting activity of [2-^{14}C]diethyl malonate. 4-hydroxy[4-^{14}C]phenylbutazone was isolated from fraction 13 in approximately 20% yield and the specific activity was found to be 248 μCi/mmole in reasonable agreement with the value of 258 μCi/mmole found for the phenylbutazone fraction.

Experimental

Throughout the synthesis dried reagents were used and precautions were taken to exclude moisture.

Inactive materials

All solvents were of AnalaR grade. Commercial phenylbutazone was recrystallized twice from absolute ethanol. Diethyl malonate and n-butyl bromide were purified by fractional distillation. Hydrazobenzene was recrystallized twice from absolute ethanol, washed with ethanol, dried in vacuo and stored under nitrogen. Sodium ethoxide solution was prepared by reaction of clean sodium metal (4.9 g) and "superdry" alcohol (75 ml) in a nitrogen atmosphere. The solution was standardized by titration with 0.1M hydrochloric acid solution using methyl orange as indicator.

Butylation of diethyl malonate

[2-^{14}C]diethyl malonate (0.039 g; 0.24 mmole: 1.5 mCi) was transferred in solution in petroleum spirit (b.p. 30–40 °C) from its containers into a 25 ml pear-shaped flask containing inactive diethyl malonate (0.760 g; 4.76 mmole). The solvent was evaporated at room temperature in a gentle stream of dry nitrogen. N-butyl bromide (0.685 g; 5 mmole) was weighed into the flask and hydrazobenzene (0.920 g; 5 mmole) and sodium iodide (0.003 g) added. The flask was fitted with a reflux condenser closed with a "Neoprene" serum cap. Dry nitrogen was passed into the flask through a long syringe needle (21 gauge), and a second short syringe needle acted as an outlet. The reactants were stirred using a 6 mm "Spinfin" magnetic stirrer[3] and the flask was heated in an oil-bath at 70–80 °C. The calculated volume (4.1 ml) of sodium ethoxide solution (2.46M; 10 mmole) was added dropwise over 3 hrs (1st 2 ml in 2 hrs, 2nd 2 ml in 1 hr) through the serum cap from a Cornwall hypodermic syringe[6] fitted with a long needle. After the addition the temperature of the heating bath was increased to 90 °C. Gentle refluxing began and this was continued for 2.5 hrs then the apparatus was sealed under nitrogen and allowed to cool overnight.

Ring closure

The apparatus was converted for distillation under nitrogen and the ethanol distilled off over one hour, with gradual increase in the temperature of the heating bath to 120 °C. The reaction mass set solid and heating was then continued at 150 °C for 2 hrs.

Isolation of phenylbutazone

Using alternate portions of water (total 20 ml) and chloroform (total 4 ml) the entire contents of the flask were transferred by Pasteur pipette into a 50 ml separating funnel, and the chloroform layer separated. The aqueous layer was extracted

with chloroform (6 x 4 ml), ether (1 x 5 ml) and run into a second 50 ml separating funnel. Acidification of the aqueous layer with 2M hydrochloric acid to pH 2 gave a yellow precipitate which was extracted into chloroform (6 x 4 ml). The extracts were combined, solvent rotary evaporated and the residue A (1.45 g) dried in vacuo. TLC analysis followed by radiochromatogram scanning showed the presence of radioactive phenylbutazone, together with at least three other active compounds and at least two inactive compounds. Phenylbutazone, however, represented approximately 95% of the total activity.

Table 1
Analysis of column eluent

Fraction	Colour	Compound[a]	Total activity, μCi
1– 3	–	–	–
4– 5	yellow	azobenzene	–
6	–	–	–
7–12	–	PB	917
13	yellow	PB, HPB, U	263
14	yellow	PB, HPB, U	14
15–16	yellow	HPB, U	4

[a]PB – phenylbutazone.
HPB – 4-hydroxyphenylbutazone.
U – one or more unidentified compounds.

Purification of phenylbutazone

The product A was dissolved in a minimum volume of chloroform and the solution applied to the top of a column (85 x 2.5 cm) of silica gel [Mallinckrodt SilicAR CC4 (100–200 mesh)]. The column was eluted with a mixture of cyclohexane and chloroform (1 : 1, v/v). Fractions (100 ml) of eluent were collected and analysed by TLC (see Table 1).

The first band to emerge was yellow and contained azobenzene. Phenylbutazone was eluted next and the colourless eluent (fractions 7–12) was collected in one portion until the emergence of the second yellow band. Further fractions were collected until the absence of solute was shown by TLC. Solvent was removed from the fractions by rotary evaporation at 50 °C. Fractions 7–12 yielded phenylbutazone (1.09 g; 71%), product B, which was dried in vacuo. Fraction 13 gave a yellow residue (0.22 g) which contained 4-hydroxy-[4–^{14}C]phenylbutazone.

Dilution of [4-^{14}C] phenylbutazone

Product B (1.092 g) and pure inactive phenylbutazone (2.217 g) were dissolved in warm ether and after equilibration phenylbutazone crystallized at -30 °C. A second recrystallization gave product C which had a specific activity of 89.6 μCi/mmole.

The combined mother liquors were evaporated to dryness at room temperature in a nitrogen stream and to the residue (1.059 g) was added further inactive phenylbutazone (2.892 g). Low temperature recrystallization from ether (x3) gave product E which had a specific activity of 23.9 μCi/mmole.

The radiochemical purities of products C and E were demonstrated by reverse isotope dilution analysis. Product C (26.71 mg) and inactive phenylbutazone (973.2 mg) were equilibrated in warm ethanol and crystallized at -30 °C. The product was then recrystallized to constant specific activity, firstly from petroleum ether (b. p. 40-60 °C), then from ether, to give product C' (195 mg). A sample of product E (29.52 mg) and inactive phenylbutazone (996.3 mg) were treated in the same manner to give product E' (72 mg). The observed specific activities of compounds B, C, E, C' and E' are recorded in Table 2 and may be compared with values calculated from the appropriate parent values and dilution factors.

The radiochemical purities of C and E are 98% with an uncertainty of approximately 2% due to the assay technique. TLC of product C followed by radiochromatogram scanning and calculation of peak areas showed the radiochemical purity to be 98%, with 4-hydroxy [4-^{14}C] phenylbutazone representing 2% of the total activity. The TLC spots were also assayed by liquid scintillation counting. Each spot was scraped into a counting vial, toluene and scintillation solution added and the mixture gently shaken. By this procedure only approximately 75% of the activity originally applied to the plate was counted. This is in agreement with observations that phenylbutazone is eluted with difficulty from silica gel.[7] Estimates of radiochemical purity by this method are thus unreliable.

4-hydroxyphenylbutazone

Phenylbutazone (10 g) was heated in boiling acetone (125 ml) under a reflux condenser for 24 hrs and the solution then allowed to stand at room temperature for

Table 2
Radiochemical assay of [4-^{14}C] phenylbutazone

Sample	B	C	E	C'	E'
Observed activity, μCi/mmole	258	89.6	23.9	2.42	0.671
Calculated activity, μCi/mmole	-	85.6	24.0	2.47	0.687

5 days. The acetone was distilled off and the residue was dissolved in ether (200 ml). The ether solution was extracted with 10% sodium carbonate solution (5 x 200 ml), then dried over anhydrous magnesium sulphate. The ether was evaporated in a nitrogen stream, leaving a yellow residue (0.33 g). TLC showed that the residue consisted mainly of the oxidation product with only traces of phenylbutazone and other compounds present. The residue was dissolved in methanol (2 ml) and the solution added to water (200 ml). A white precipitate (0.132 g) was filtered off and dried in vacuo. Microanalysis was performed on a Perkin Elmer model 240 Analyser,

Found: C, 69.5; H, 6.4; N, 8.4%, Calculated for $C_{19}H_{20}N_2O_3$

C, 70.4; H, 6.2; N, 8.6%, m.p. 131 °C; literature m.p. 131–2 °C.[4, 5]

The mass spectrum was recorded using a GEC-AEI MS 30 double beam mass spectrometer, the infrared spectrum using a Perkin-Elmer Model 325 Infrared Spectrophotometer, and the proton magnetic resonance spectrum at 60 MHz using a Perkin-Elmer R12 NMR spectrometer.

4-hydroxy [4-^{14}C] phenylbutazone

TLC analysis followed by radiochromatogram scanning showed that column fraction 13 (220 mg; 260 μ Ci) contained approximately 60% phenylbutazone (130 mg; 160 μ Ci) and 20% 4-hydroxy[4-^{14}C]phenylbutazone (40 mg; 50 μ Ci). To remove the bulk of the [4-^{14}C]phenylbutazone, fraction 13 was dissolved in ether (10 ml) and extracted with 10% aqueous sodium carbonate solution (5 x 20 ml). The ether solution was filtered, dried over anhydrous magnesium sulphate, and evaporated using a nitrogen stream to leave a yellow residue (28 mg). The residue was then dissolved in chloroform (1 ml) and the solution applied to two silica gel TLC plates (20 x 20 cm; 0.25 mm). Triple development in the solvent system chloroform-acetone (9 : 1 v/v) separated 4-hydroxy [4-^{14}C]phenylbutazone (R_f 0.21) from phenylbutazone (R_f 0.71) and two yellow compounds (R_f 0.27, 0.25). The 4-hydroxy [4-^{14}C]phenylbutazone bands were scraped from the TLC plates into a Gooch crucible and the silica gel eluted with portions of ether. The combined filtrates were evaporated to dryness in a nitrogen stream and the residue (10 mg; 0.031 mmole) dried in a desiccator over phosphorus pentoxide.

TLC analysis followed by radioscanning showed the radiochemical purity of the product to be >95%. Azobenzene was detected in the analysis showing that some degradation of the product had occurred. The specific activity of the 4-hydroxy [4-^{14}C] phenylbutazone was 248 μ Ci/mmole, and the total activity was 8 μ Ci.

Thin-layer chromatography

Merck pre-cast fluorescent silica gel GF_{254} plates were used. Solvent systems were (1) cyclohexane-chloroform-methanol-glacial acetic acid (12 : 6 : 1 : 1),[8] (2) chloroform-acetone (9 : 1),[7] and (3) petroleum spirit (b. p. 40–60 °C) diethyl ether (1 : 1). Samples were 5–20 μl of a freshly prepared 1% solution in ether or

Table 3
R_f values

Solvent system	4-hydroxy phenylbutazone	Phenyl butazone	Hydrazo benzene	Azobenzene
1	0.26	0.48	0.48	0.65
2	0.40	0.59	0.61	0.67
3	0.18	0.33	0.50	0.60

chloroform. Compounds were detected by exposing the plate to ultraviolet light of wavelength 254 mμ, or by spraying with a 1% aqueous solution of ammonium metavanadate, then with 2.5M sulphuric acid, and then warming the plate at 120 °C for 1 min.[7] Approximate R_f values after single development are recorded in Table 3.

Radiochemical assay

Activities were determined by liquid scintillation counting using a Packard TriCarb Liquid Scintillation Counter. A sample of product (1×10^{-2} - 2×10^{-4} g) was dissolved in toluene and made up to 100 ml of solution. For counting, 2 ml portions of these toluene solutions were transferred to 20 ml counting vials and made up to 10 ml with 3 ml of toluene and 5 ml of a 1% solution of butyl-PBD in toluene. Counting efficiencies (~88%) were determined by the channels ratio method and errors (standard deviation) were approximately ±1%. Radiochromatogram scanning was by means of a Panax model RTLS - 1A scanner and efficiency of detection of ^{14}C was very approximately 10%. Radiochemical purities were estimated by comparison of peak areas, which were calculated by approximation of the peaks to triangles.

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