PREPARATION OF CARBON-14 LABELLED ETHYL 2-(P-CHLOROPHENOXY) -2-METHYL PROPIONATE (CLOFIBRATE; ATROMID-S*); CLOFIBRIC ACID AND CLOFIBRIC AMIDE

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SUMMARY

The preparation of [^{14}C] Ethyl 2-(p-chlorophenoxy)-2-methyl propionate (Atromid-S; clofibrate) with the label separately located in three different positions is described, together with purification procedures employed. From each of the three labelled products, clofibric acid (CPIB acid;ICI 7132) has been prepared by hydrolysis. The labelled amide has also been synthesised.

Key Words: Carbon-14, clofibrate, synthesis, autoradiography, clofibric acid.

INTRODUCTION

Since its introduction in 1962 clofibrate [Ethyl 2-(4-chlorophenoxy)-2-methyl propionate] has become one of the most widely used anti-hyperlipidemic agents. Thorp[1] showed it to be rapidly hydrolysed by tissue and serum esterases to clofibric acid (CPIB) (see Scheme 3) which is the pharmacologically active metabolite.

In 1975 Ferdinandi[2] prepared [^{14}C]clofibrate in the C_2 position. The present paper describes earlier syntheses which were designed to incorporate

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the isotopic label in three separate positions [C_1 (Scheme 1); C_2-methyl (Scheme 2) and in the p-chlorophenyl ring]. An aliquot of the clofibrate from each labelled synthesis has been hydrolysed to produce the corresponding ^{14}C labelled clofibric acid. A synthesis of the clofibric amide [2-(p-chlorophenoxy)-2-methyl[1-^{14}C]propionamide] for metabolic investigation, is also described.

When orally administered to man [^{14}C] clofibrate was found to be completely absorbed, 99% of the radiolabelled dose was accounted for in the urine[3], and no radioactivity was detected in the faeces. Although no conjugated clofibric acid has been detected in human plasma, at least 60% is conjugated in the urine mainly as the glucuronide[4]. In rats [^{14}C] clofibrate and [^{14}C] clofibric acid when administered in equimolar oral doses show essentially the same profiles in blood levels, tissue distribution and excretion[5]. Examination of the serum showed that all the radioactivity was due to clofibric acid (CPIB) and in urine CPIB was found free and as the glucuronide conjugate. In dogs, however, the same pharmacological equivalence of CPIB and clofibrate was not demonstrated since the serum contained 40% more radioactivity after an oral dose of [^{14}C] clofibric acid than after an equimolar dose of [^{14}C] clofibrate[5].

Caldwell[6] dosed [^{14}C] clofibric acid to rats, guinea pigs, rabbits, cats, dogs, ferrets and man. The percentage of [^{14}C] in the 0 - 24 hour urine was compounded of the free acid and glucuronide. The taurine conjugate was also found in 3 species.

	Rat	Guinea Pig	Rabbit	Dog	Cat	Ferret	Man
Free Acid	44	20	9	30	56	23	5
Glucuronide	56	80	91	19	-	5	95
Taurine Conjugate	-	-	-	51	43	72	-

MATERIALS

[1-^{14}C]Sodium isobutyrate, [^{14}C] methyl iodide and [U-^{14}C]-p-chlorophenol were obtained from Amersham International plc. Isobutyric acid was purchased from Koch-Light Ltd. All samples used in the radiochemical purity and specific activity determinations were prepared in standard glass screw-cap vials of low potassium content (Packard Instruments Ltd., Wembley) and counted on a Packard Tri-Carb Liquid Scintillation Spectrophotometer model 3320 or model 314. The 2,5-diphenyl oxazole (P.P.O.) and 1,4-bis[2-(4 methyl-5-phenyloxazoyl)]benzene (DM POPOP) were obtained from Packard Instruments Ltd. Naphthalene (scintillation grade) was purchased from Thorn Electronics Ltd. Autoradiographic studies employed either Kodak 'Kodirex' or Agfa 'Structurix' X-ray film. Sulphur free toluene (May and Baker Ltd.) was used without further purification. Other solvents used, were either redistilled or of analytical reagent quality. The plates used for chromatography (TLC; preparative TLC) were either prepared from Merck Silica GF, or were purchased from Anachem Ltd. The silica film was 0.25 mm thick for all analytical determinations and 0.5 mm for preparative purifications.

THIN LAYER CHROMATOGRAPHY(TLC)

Seven systems were used throughout this work; these were

System A; Silica GF developed with benzene.

System B; Silica GF developed with petroleum ether (Br 100 - 120°C); acetone [90:10].

System C; Silica GF developed with toluene.

System D; Silica GF developed with petroleum ether (Br 40 - 60°C); acetone [50:1].

System E; Silica GF developed with petroleum ether (Br 100 - 120°C); ethyl acetate [2:1].

System F; Silica GF developed with toluene; dioxan; acetic acid [90:25:4].

System G; Silica GF developed with n-butanol; acetic acid; water [40:10:5].

System H; Silica GF developed with benzene; ethyl acetate [50:50].

EXPERIMENTAL

LABEL No. 1 (SCHEME 1)

PREPARATION OF ETHYL 2-[P-CHLOROPHENOXY]-2-METHYL-[1-^{14}C]PROPIONATE

Stage 1

Conversion of [1-^{14}C]Sodium Isobutyrate to [1-^{14}C]Isobutyric Acid

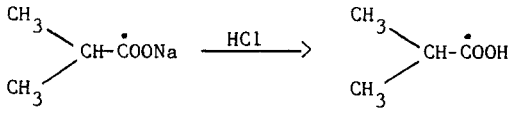
Commercially available unlabelled isobutyric acid used for isotopic dilution of the [1-^{14}C] isobutyric acid was found to contain 1 - 1.5% of n-butyric acid. The full synthesis using unlabelled intermediates had shown the n-butyric acid impurity to react with the reagents used in the synthesis to yield p-chlorophenoxy-n-butyric acid ethyl ester as a contaminant in the final product, which was extremely difficult to remove. The commercially available isobutyric acid was therefore purified by Autoprep GLC, prior to use in the labelled synthesis.

[1-^{14}C] Sodium isobutyrate (3.5 mCi) was dissolved in distilled water (4.0 ml) and transferred to a separation funnel, acidified with 1.0M hydrochloric acid solution (4.0 ml), and then extracted with diethyl ether (5 x 1.5 ml). The aqueous phase was scavenged by the addition of purified unlabelled isobutyric acid (1.1 g) and serially extracted with diethyl ether (5 x 15 ml). The total ether extracts were combined and dried over anhydrous magnesium sulphate for 16 hours. After filtration, the ether extract was evaporated to dryness under reduced pressure to yield a clear oil (1.38 g).

PREPARATION OF ETHYL-2-[P-CHLOROPHENOXY]-2-METHYL-1-[^{14}C]PROPIONATE

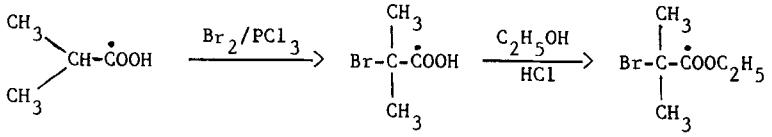
SCHEME 1

Stage 1



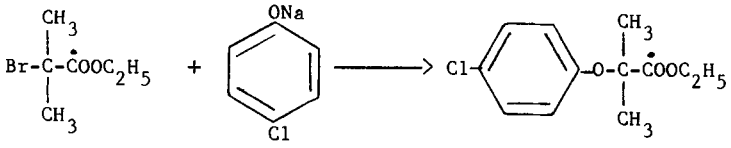
(MW 88)
(1)

Stage 2



(MW 88) (1) (MW 167) (MW 195) (2)

Stage 3



(MW 195) (2) (MW 242.5) (3)

• Indicates position of ^{14}C label

Stage 2

PREPARATION OF ETHYL 2-BROMO-2-METHYL-1-[^{14}C]PROPIONATE (2)

[1-^{14}C] Isobutyric acid (1.38 g) was stirred under anhydrous conditions and bromine (1.3 ml) was added dropwise over 15 minutes. Phosphorus trichloride (0.5 ml) was also added dropwise over 3 minutes and the mixture stirred in a silicone bath at 70 - 75°C for 5 hours. The bath temperature was raised to 100°C and the mixture stirred for a further 1 hour to remove the excess bromine. To the yellow brown solution absolute ethyl alcohol saturated with hydrogen chloride (8.0 ml) was added and stirred at ambient temperature for 16 hours. The mixture was treated with crushed ice and serially extracted with diethyl ether (7 x 20 ml). The combined ether extracts were washed with 2% aqueous sodium hydroxide (2 x 20 ml) followed by water (2 x 10 ml) and dried over anhydrous magnesium sulphate. After filtration the ether extracts were evaporated to dryness under reduced pressure at ambient temperature to produce a light-brown oil (2.04 g; stage yield 66.8%).

Stage 3

PREPARATION OF ETHYL 2-(P-CHLOROPHENOXY)-2-METHYL-1-[^{14}C]-PROPIONATE (3)

p-Chlorophenol (1.3 g) was stirred with freshly distilled xylene (8.0 ml) and sodium hydride [5% dispersion in oil] (0.5 g) was added and the stirred mixture heated at 160°C for 20 minutes. The bath temperature was reduced to 120 - 130°C and a solution of ethyl 2-bromo-2-methyl-1-[^{14}C]propionate (2.0 g) in dried xylene (3.0 ml) was added dropwise over five minutes. The bath temperature was then raised to 160°C and the reaction mixture stirred and refluxed for 4 hours. The mixture was cooled, distilled water (10 ml) was added and the xylene-water mixture extracted with diethyl-ether (15 ml). The gelatinous emulsion so formed was slow to break, and the

aqueous phase was then removed. The xylene-ether phase was serially extracted with 10% aqueous sodium hydroxide solution (3 x 50 ml) to remove excess p-chorophenol, followed by a distilled water wash (10 ml), before drying over anhydrous magnesium sulphate. The extract was examined by TLC (System A) and visualisation under UV 254 nm showed that the required product had been formed, together with several impurities. Autoradiography showed that all the components in the chromatographic separation were radiolabelled.

The dried xylene-ether extract was filtered, and the extract, together with the ether washings of the drying agent was evaporated under reduced pressure from a water bath at 25°C, to remove the ether. The water bath temperature was then raised to 60°C, and the evaporation continued until all the xylene had been removed. A yellow-brown oil was obtained (2.25 g; stage yield 87%).

The product was examined by GLC and showed predominantly the required product; p-chlorophenoxy n-butyric acid ethyl ester was not detected. Other impurities including residual traces of xylene were found. By GLC, the product purity was 87%.

PURITY

The crude product was purified on a 1" diameter column packed with Merck Silica GF (40 g), which was washed with AR benzene for 5 hours before application of the crude product dissolved in benzene (2.0 ml).

Fractions (1.0 ml) were collected and 1 microlitre aliquots of each fraction were chromatographed by TLC (System A) and examined by UV 254 nm and autoradiography. It was found that fractions 76 - 118 contained only [^{14}C] Clofibrate with identical R_f to pure reference material.

The fractions were combined and evaporated to dryness under reduced pressure from a water bath at 50°C, a pale yellow oil was obtained (1.13 g. Overall chemical yield 29.7%). The chemical purity by GLC was shown to be 99.2%. Examination by TLC on systems A and B against pure reference clofibrate and visualisation under UV 254 nm showed only the required product. This was confirmed by autoradiography. The autoradiograph was used to "map" the plate which was then segmented and counted. Radiochemical purities of 99.5% on system A and 99.3% on system B were obtained..

The specific activity was determined in duplicate on each of two solutions and was shown to be 1.1 μCi/mg [0.27 mCi/mmol] which represents a 35% overall radiochemical yield for the synthesis.

LABEL No. 2 (SCHEME 2)

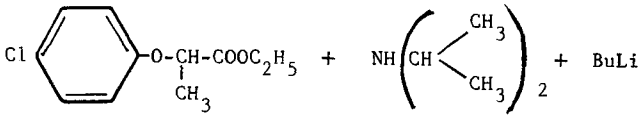
PREPARATION OF ETHYL 2-(P-CHLOROPHENOXY)-2-[^{14}C]-METHYL PROPIONATE (2)

The tetrahydrofuran used was dried and distilled over lithium aluminium hydride immediately prior to use.

Tetrahydrofuran (THF) (2.0 ml) was stirred in a flask at -65°C, immersed in a solid carbon dioxide-acetone bath in an atmosphere of argon and both the temperature and inert atmosphere were maintained throughout the synthesis. Di-isopropylamine (363 mg; 3.6 mmol) was added and the mixture stirred for 15 minutes before adding butyl lithium in hexane (1.7 ml, 3.6 mmol). The mixture was stirred for a further 15 minutes before adding a solution of ethyl 2-(p-chlorophenoxy)propionate (1) [685 mg 3.0 mmol] in THF (3.0 ml) and stirred for 20 minutes at -65°C, when [^{14}C] methyl iodide (47 mg) together with unlabelled methyl iodide (589 mg) was introduced. The [^{14}C] methyl iodide was transferred on a vacuum line to the unlabelled material, and the whole transferred to the reaction vessel via the vacuum

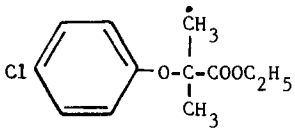
PREPARATION OF ETHYL 2-(P-CHLOROPHENOXY)-2-[^{14}C]-METHYL PROPIONATE

SCHEME 2



(1) (MW 228.5)

(MW 142)



(MW 242.5) (2)

• Indicates the position of the ^{14}C label

line. When the transfer was completed the mixture was stirred at -65°C for 20 minutes before removing the cooling bath and allowing the reaction mixture to slowly warm to ambient temperature. Hydrochloric acid 3M (10 ml) was added and stirred for 30 minutes before serially extracting with diethyl ether (8 x 20 ml). The combined ether extracts were washed with water (3 x 20 ml) and dried over anhydrous magnesium sulphate. After filtration the ether extract was evaporated to dryness to yield a light brown oil (740 mg). The crude product was examined by gas chromatography-mass spectrometry using an LKB 9000 spectrometer with a 1% OV-1 column, an oven temperature of 140°C and a helium carrier gas flow at 30 ml/min. The required product was the major component, but small amounts of both the methyl and propyl ester were also detected.

PURIFICATION

The crude product was dissolved in absolute ethyl alcohol and equal aliquots applied as thin streaks to 16 Merck Silica GF PTLC plates which were then developed with system B. The bands corresponding in R_f to reference clofibrate were removed and the product extracted from the silica by stirring with absolute ethyl alcohol for 16 hours at ambient temperature. The ethyl alcohol extract was freed from the silica by filtration through a pad of High-Flow Supercell and evaporated to dryness under reduced pressure from a water bath at 40°C. A light brown oil (618 mg) was obtained. The overall yield based on methyl iodide was 56.9%. A series of concentrations of the product in absolute alcohol were applied to two silica plates which were developed in solvent systems A and B. Visualisation under UV 254 nm showed only the required product. Autoradiography confirmed that the chemical and radiochemical chromatographic separations were identical. The autoradiographs were used to "map" the TLC plates which were segmented and

counted to show a radiochemical purity of 99.0% on system A and 99.5 % on system B. GLC examination of the product using a 5% SE 30 column with an oven temperature of 180°C showed predominantly a single peak identical to reference clofibrate. Triplicate determinations on each of two solutions showed the specific activity to be 7.3 μCi/mg (1.8 mCi/mmol).

LABEL No. 3

PREPARATION OF ETHYL 2-([U-^{14}C]-P-CHLOROPHENOXY)-2-METHYL PROPIONATE

[U-^{14}C]-p-chlorophenol (32.1 mg; 5 mCi) was isotopically diluted at ambient temperature with unlabelled p-chlorophenol (225.2 mg) and stirred at ambient temperature with dried, freshly distilled xylene (3.0 ml) for 30 minutes. Sodium hydride (50% dispersion in oil) (106.5 mg) was added and stirred a for 30 minutes at ambient temperature. The reaction mixture was then stirred in a silicone bath for 20 minutes at 115 - 120°C before cooling and adding a solution of ethyl 2-bromo-2-methyl propionate (600 mg) in dried xylene (1.0 ml) and heating for 4 hours at 150 - 160°C. The mixture was then cooled, added to water (10 ml) and extracted with diethyl ether (5 x 10 ml). The combined ether extracts were washed with a 10% aqueous sodium hydroxide solution (2 x 20 ml) followed by a water wash (5 x 10 ml) and then dried over anhydrous magnesium sulphate. The ether extract was examined by TLC (System C), and showed "one spot" material identical with pure reference clofibrate when viewed under UV 254 nm. This result was confirmed by autoradiography.

PURIFICATION

The dried ether extracts were filtered, and the filtrate together with the ether washings were evaporated to dryness under reduced pressure at ambient temperature. The residue was dissolved in AR acetone (4.0 ml)

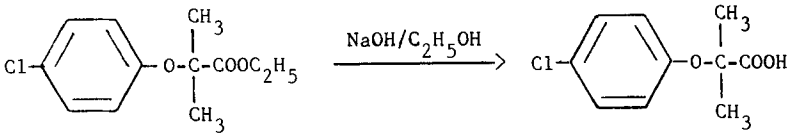
and equal aliquots applied to eight Merck Silica GF PTLC plates, and developed with solvent system C. The bands corresponding in R_f to reference clofibrate were removed and the product extracted from the silica by stirring with AR acetone for 16 hours at ambient temperature. The silica was removed by filtration and the acetone extract and washings were evaporated to dryness under reduced pressure. The residue obtained was dissolved in absolute ethyl alcohol and centrifuged to separate traces of silica. The clear extract was evaporated to dryness in a nitrogen stream and dried under reduced pressure at ambient temperature for 16 hours, to yield an orange oil (296.4 mg) representing an overall yield of 61% based on p-chlorophenol.

PURITY CRITERIA

The product was examined by TLC employing systems A, D and E. and in each system only one component, which corresponded in R_f to reference clofibrate was seen when viewed under UV 254 nm. Autoradiography confirmed that the radiochemical and chemical separations were identical. The plates were segmented and counted and duplicate determinations carried out for each of the three chromatographic systems used. The radiochemical purity from the six determinations was 99.7 $\pm$ 0.04%. The specific activity was determined in duplicate on each of two solutions and shown to be 12.2 μCi $\pm$ 0.20 μCi/mg [2.96 mCi/mmol]. The product was examined by gas chromatography-mass spectrometry (GCMS) using an LKB 9000 and employing an OV-1 column, oven temperature 136°C. A single peak identical to reference material was the sole component. Elemental analysis showed C 59.4; H 6.2: $C_{12}H_{15}O_3Cl$ requires C59.8; H 6.5.

PREPARATION OF 2-(P-CHLOROPHENOXY)-2-METHYL PROPIONIC ACID (CLOFIBRATE ACID)

SCHEME 3



(1) (2)

(MW 242.5) (MW 214.5)

PREPARATION OF 2-(P-CHLOROPHENOXY)-2-METHYL PROPIONIC ACID (CLOFIBRIC ACID) (2)

SCHEME 3

For each of the preparations of clofibrate where the [^{14}C] label was incorporated in three separate molecular positions an aliquot was hydrolysed to produce 2-(p-chlorophenoxy)-2-methyl propionic acid (2), ICI 7132; (clofibric acid). The conditions of hydrolysis and purification were as follows. Ethyl 2-(p-chlorophenoxy)-2-methyl propionate (1.0 mmol), methylated spirits 74 OP (14.5 ml) and sodium hydroxide (17.0 mmol) were stirred at ambient temperature for 24 hours. The solution was evaporated to dryness under reduced pressure at ambient temperature, and water (15 ml) was added before extracting with ether (4 x 5.0 ml) to remove any unreacted ester. The ether extracts were then back extracted with water (2 x 5.0 ml) and the combined aqueous extracts were cooled in an ice/water bath before acidifying with 1.0M hydrochloric acid and extracting with diethyl ether (5 x 20 ml). The combined ether extracts were dried over anhydrous magnesium sulphate for 16 hours. After filtration, the ether extracts and washings were evaporated to dryness under reduced pressure to produce a white crystalline solid (yield 78.4%).

PURITY CRITERIA

A 2 mg% solution of [^{14}C]clofibric acid in AR methanol was prepared and a gradient of concentrations applied to two silica GF plates which were developed in solvent systems F and G. Visualisation under UV 254 nm showed only one component identical in R_f with pure reference clofibrate acid. Autoradiography confirmed that the product was radioactive, and no other components were detected. The autoradiograph was used to "map" the plate which was segmented and each chromatographic strip was counted. The radiochemical purity was shown to be 99.7% ± 0.36% for the four determinations. Elemental analysis showed C 55.9%; H 5.2%: $C_{10}H_{11}O_3Cl$ required C 55.9% and H 5.1%. The product was examined by gas chromatography-mass spectrometry using an LKB 9000 with an OV-1 column and an oven temperature of 129 and 210°C. Only the required clofibric acid was seen. The product was also examined by high performance liquid chromatography using a spherisorb ODS 20 cm column eluted with a solvent mixture of methanol 70%; water 30%; trifluoroacetic acid 0.1% and a flow rate of 1.5 ml/min. The UV wavelength was 229 nm and a 10 µl injection sample of a 1.0 mg/ml solution in the developing solvent was used. Fractions (1.0 ml) were collected and counted in phosphor (10 ml). The radiochemical purity by HPLC was shown to be 99.9%.

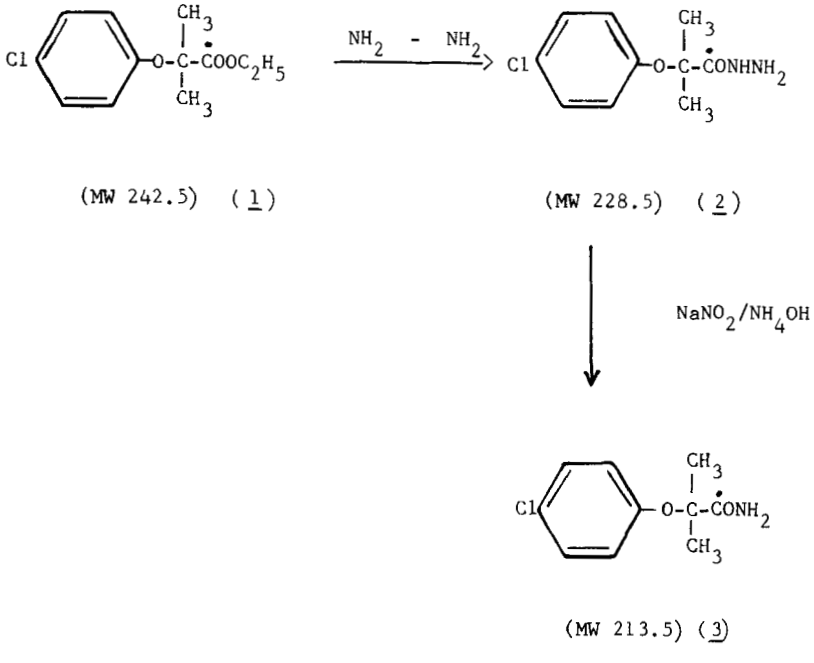
PREPARATION OF THE AMIDE OF CLOFIBRATE 2-(P-CHLOROPHENOXY)-2-METHYL [1-^{14}C] PROPIONAMIDE (ICI 40,979)

The clofibric amide was prepared via the synthetic pathway shown in Scheme 4. Ethyl 2-(p-chlorophenyl)-2-methyl[1-^{14}C] propionate (150 mg; 158 µCi) together with hydrazine-hydrate (64%; 0.15 ml) and ethyl alcohol 74 OP (1.5 ml) were stirred and refluxed for 10 hours. The oily emulsion obtained was extracted with diethyl ether (4 x 10 ml) and the

extracts were combined and dried for 16 hours over anhydrous magnesium sulphate. After filtration the combined ether extracts and washings were evaporated to dryness under reduced pressure when a colourless oil was produced which slowly crystallised on standing (104 mg; 73.6% stage yield).

PREPARATION OF THE AMIDE OF CLOFIBRATE 2-(P-CHLOROPHENOXY-2-METHYL [1-^{14}C] PROPIONAMIDE (ICI 40,979)

SCHEME 4



(MW 242.5) (1)

(MW 228.5) (2)

(MW 213.5) (3)

• Indicates the position of the ^{14}C label

Examination by TLC (system H) and visualisation under UV 254 nm showed the product to be predominantly the required material with identical R_f to reference clofibric acid hydrazide. This was confirmed by autoradiography. The clofibric acid hydrazide (104 mg) was stirred in 1.0M hydrochloric acid solution (0.7 ml), diethyl ether (1.0 ml) was added and the mixture stirred and cooled to 0 - 5°C surrounded by an ice-water bath before adding a solution of sodium nitrate (31.5 mg) in water (0.2 ml). After the addition, the mixture was stirred for a further 30 minutes at 0 - 5°C before allowing to warm to ambient temperature and maintaining stirring for an additional 45 minutes. The ether layer was removed and the aqueous phase serially extracted with diethyl ether (5 x 2.0 ml). The combined ether extracts were washed with a 5% aqueous solution of sodium bicarbonate (2 x 10 ml) before drying over anhydrous magnesium sulphate for 16 hours. After filtration to remove the drying agent, the ethereal solution was treated with 0.880 ammonium hydroxide solution (3.0 ml) and maintained at ambient temperature for 24 hours. On evaporation under reduced pressure a white semi-crystalline solid was obtained (82.8 mg; 85% stage yield). The overall chemical yield was 62.7%.

Examination of the product by TLC (sytem H) showed only a single component, when viewed under UV 254 nm, which was identical in R_f to pure reference clofibric amide. Autoradiography confirmed this result. The radiochemical purity using TLC system H was shown to be 99.2%. Elemental analysis showed C 56.1; H 5.9; N 6.8: $C_{10}H_{12}O_2NCl$ requires C 56.2; H 5.6; N 6.6.

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