SYNTHESIS AND PURIFICATION OF CARBON-14 LABELLED 1, 1-HEXAMETHYLENE-BIS [5-(4-CHLOROPHENYL)BIGUANIDE] (CHLORHEXIDINE, 'HIBITANE'*)

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SUMMARY

Two syntheses of [^{14}C] chlorhexidine ('Hibitane'*) with the label specifically incorporated in two separate molecular positions are described. Ring labelled chlorhexidine prepared from p-chloro[U-^{14}C]aniline was obtained with a molar specific activity of 27.9 mCi/mmol. Chain labelled material, where the ^{14}C label was incorporated in the 1 and 6 positions of the hexamethylene bridge, was prepared from [1, 6 ^{14}C]-adiponitrile, with a molar specific activity of 11.5 mCi/mmol. Several methods of purification are described.

Key Words: Chlorhexidine, carbon-14, purity.

INTRODUCTION

Chlorhexidine (Hibitane*) is one of a number of poly-biguanides investigated during the late 1940's and early 1950's in ICI laboratories for anti-bacterial activity.(1) It was shown to possess outstanding bacteriostatic properties against gram-positive bacteria, even at high dilutions, and to be compatible with penicillin, streptomycin, oxytetracyclin and aureomycin. In the veterinary field it has been used extensively as an udder wash. Throughout the last twenty years chlorhexidine has been widely used clinically as a topically applied bactericide for skin infection, wounds, burns, obstetrics and bladder irrigation. The anti- bacterial properties of chlorhexidine have been reviewed by Hennessey (3).

* Hibitane, trade mark the property of Imperial Chemical Industries PLC.

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In 1969 Löe (2) demonstrated that a one minute mouth rinse twice daily with a 0.2% aqueous solution of chlorhexidine gluconate completely inhibited the formation of dental plaque, and since then other investigators have studied the use of chlorhexidine in oral hygiene.

Radiolabelled compounds were not so readily available thirty years ago and consequently absorption, distribution,metabolism and excretion studies were not performed in any depth at the time. The [^{14}C] chlorhexidine containing the radioisotope in two separate molecular positions was prepared so that such studies could be undertaken (4). The two positions labelled were (a) in the aromatic rings of the p-chloroaniline moieties, and (b) the 1 and 6 carbon atoms of the hexamethylene bridge. These are designated Ring and Chain labelled respectively.

Chlorhexidine was discovered before the advent of thin layer chromatography (TLC) and in view of its binding properties to most adsorbents, considerable investigation was needed before suitable TLC systems (A and B) were found. Further complications were encountered upon endeavouring to purify the product by TLC on a preparative scale in that only 4% of the chlorhexidine bound to the silica could be removed by extraction with polar organic solvents. In both syntheses, the product was isolated as the base, and stored, after purification, at 0° - 4°C. For some studies the solubility of [^{14}C] Chlorhexidine was enhanced by conversion to the gluconate salt.

MATERIALS

p-Chloro[U-^{14}C]aniline and potassium [^{14}C]cyanide were purchased from Amersham International plc. Commercially available anhydrous diethyl ether was additionally dried over sodium wire. Sulphur free toluene (May and Baker Ltd.)

was used without further purification. All other solvents used were either of analytical reagent quality or were redistilled. Lithium aluminium hydride was purchased from British Drug Houses Ltd. and adiponitrile from Aldrich Chemical Company Ltd. All samples used for the determination of radiochemical purity and specific activity were counted on a Packard Tri Carb Liquid Scintillation Spectrophotometer model 3320 in standard glass screw cap vials of low potassium content (Packard Instruments Ltd., Wembley). The 2,5-diphenyl oxazole (PPO) and 1,4-bis [2-(4 methyl-5-phenyl oxazoyl)]-benzene (DMPOPOP) were purchased from the same source. Naphthalene (scintillation grade) was obtained from Thorn Electronics Ltd. All autoradiographic examinations employed either Kodak 'Kodirex', or Agfa 'Structurix' X-ray film.

THIN LAYER CHROMATOGRAPHY (TLC)

Chlorhexidine binds strongly to silica and glass surfaces and this has caused difficulties in analytical and purification procedures. In all chromatographic investigations Merck Silica GF or HRF was used as the adsorbent.

Three TLC Systems were used throughout this work, these were:-

System A Merck Silica GF or HRF developed with toluene;ammonia (0.880); methanol AR [60:20:70]

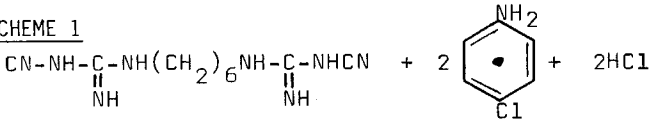
System B Merck Silica GF or HRF developed with toluene;ethyl acetate; ammonia (0.880);ethanol 740P [60:20:10:40]

System C Merck Silica GF developed with n-butanol;acetic acid;water [12:3:5]

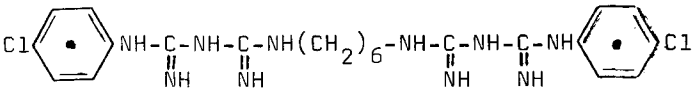
For all analytical studies the plates used had dimensions of 20 x 20 cm and the thickness of the silica layer was 0.25 mm. Preparative TLC was carried out using 20 x 40 cm plates and a silica film of 0.5 mm thickness.

PREPARATION OF RING LABELLED [^{14}C]CHLORHEXIDINE

SCHEME 1



(1) (MW 250) (2) (MW 127.5)



(MW 505) (3)

• Marks the position of the carbon-14 label

EXPERIMENTAL

p-Chloro[U-^{14}C]aniline (2) (79 mg; 16.14 mCi/mmol) was isotopically diluted with unlabelled p-chloroaniline (6.0 mg) and stirred at ambient temperature with isopropanol (2.0 ml) in the presence of 10 M hydrochloric acid (66 mg). Stirring was continued for 20 minutes before adding 1,6-hexamethylene-bis-dicyandiamide (1)(H.M.B.D.A., 83.3 mg (0.33 mmol) in isopropanol (2.0 ml). This was stirred for 8 hours in a silicone bath maintained at 85°C. The mixture was cooled in an ice-water bath to ambient temperature and sodium hydroxide solution (1.0 ml of a 2.7% aqueous solution) was added dropwise until the reaction mixture was just alkaline. The clear solution was stirred and gradually became turbid as the white granular chlorhexidine base was deposited. After stirring for 30 minutes the mixture was centrifuged to separate, and the product washed with isopropanol (2 x 1.0 ml), isopropanol-water (1:1) (2 x 1.0 ml), and distilled water (3 x 1.0 ml), before being dried under reduced pressure for 16 hours at ambient temperature, to give 121 mg of crude chlorhexidine base (Stage Yield 72%). Examination of the product by TLC (System A) revealed the presence of thirteen impurities when visualised under UV 254 nm. Autoradiography showed that all the components of the chromatogram were radiolabelled.

PURIFICATION OF CHLORHEXIDINE BASE

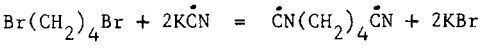
The crude base (121 mg) was dissolved in the minimum volume of the developing solvent (System A) and applied equally to nine Merck Silica HRF plates which had been pre-run with the developing solvent and dried before application of the solution of crude product. After development, the plates were dried in air, the band corresponding in R_f to reference chlorhexidine was removed, and a small column formed from the silica-chlorhexidine band. The product was eluted from the silica with the developing solvent (System A). The fractions collected were examined by TLC, selected fractions were combined and evaporated to dryness under reduced pressure from a water bath at 40°C.

The residue obtained was dissolved in absolute ethyl alcohol (3.0 ml) and allowed to crystallise at 0 - 4°C for 16 hours. The white crystalline solid was separated by centrifugation, washed with petroleum-ether (Br 60° - 80°C) (3 x 1.5 ml) and dried under reduced pressure for 16 hours at ambient temperature (42 mg; overall yield 25%). The specific activity was shown to be 55.35 μCi/mg [27.95 mCi/mmol], which represented an overall radiochemical yield of 23.2%. The radiochemical purity was 99%. To obtain material at two levels of activity, 9.0 mg of the product was retained as chlorhexidine/1R, and the remainder isotopically diluted with pure unlabelled chlorhexidine base and recrystallised from boiling absolute ethyl alcohol. The white crystalline solid obtained (chlorhexidine/2R) was washed with petroleum ether (Br 60° - 80°C) and dried under reduced pressure at ambient temperature (55.6 mg). The radiochemical purity was determined by TLC and found to be 98.9% on System B and the specific activity was 25.36 μCi/mg (12.81 mCi/mmol).

SYNTHESIS OF CHAIN LABELLED [^{14}C] CHLORHEXIDINE

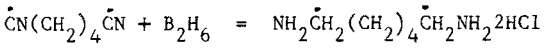
Scheme 2

Stage 1



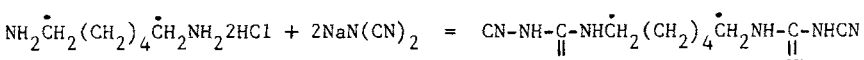
(1) (MW 216) (130) (2) (MW 108)

Stage 2



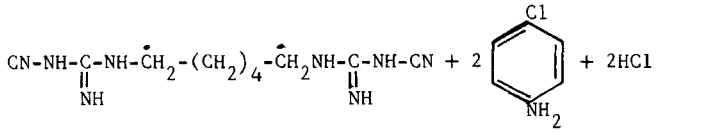
(2) (3) (MW 189)

Stage 3

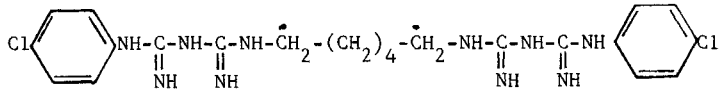


(189) (3) H.M.B.D.A. (4) (MW 250)

Stage 4



(4) (MW 127.5)



(5) (MW 505)

• Marks location of carbon-14 label

Preparation of [1, 6-^{14}C]Adiponitrile (2)

Potassium [^{14}C]cyanide (113.5 mg; 100 mCi) isotopically diluted with unlabelled potassium cyanide (406.5 mg) was stirred with dimethyl formamide (5.0 ml) which had been pre-dried and distilled over calcium hydride. To the mixture was added 1, 4-di-iodobutane (1240 mg) and stirring was continued for 16 hours at ambient temperature. Water (20 ml) was then added and the reaction mixture extracted with diethyl ether (8 x 30 ml). The ether extracts were combined, washed with distilled water (3 x 10 ml), and dried over anhydrous magnesium sulphate. After filtration, the dried extract and ether washings were evaporated to dryness under reduced pressure from a water bath at 25 - 30°C, and dried at ambient temperature under reduced pressure. Yield 380.2 mg (Stage Yield 88%). The identity of the product was established by gas-liquid chromatography (GC) on two separate columns: 5% OV 17 and 5% CM 20 at a temperature of 170°C. In each case a single peak was detected, identical to a sample of authentic adiponitrile.

Preparation of [1, 6-^{14}C]1, 6-hexanediamine dihydrochloride (3)

The tetrahydrofuran (THF) used for this stage of the synthesis was distilled over lithium aluminium hydride and stored under argon immediately prior to use.

Diborane in THF (0.78 mmol/ml; 7.2 ml) was added to [1, 6-^{14}C]adiponitrile (2) (380.2 mg) stirred in THF (5.0 ml) and stirred for 16 hours. The excess diborane in the gelatinous mixture produced, was decomposed by the dropwise addition of absolute ethyl alcohol. The hydrochloride of the product was formed by the addition of an ethereal solution of hydrogen chloride until no further precipitation occurred. The reaction liquors were evaporated to dryness under reduced pressure from a water bath at 45°C. The residue was dissolved in methanol AR, centrifuged to separate from traces of insoluble material and the clear supernatant evaporated to dryness in a nitrogen stream.

Methanol AR (3.5 ml) was added, warmed to give a clear solution and ethyl acetate added dropwise until the mixture was turbid. After 16 hours at 0 - 4°C a white crystalline solid was deposited which was separated by centrifugation and washed with sodium dried ether (3 x 4.0 ml) before drying under reduced pressure at 40°C (455.5 mg; 69% Yield).

The identity of the product was established by gas-liquid chromatography (GC). Two separate columns were used for analysis, 10% CM 20 and 5% SE 30, at 185°C. In each case a single peak was obtained showing identical retention times to authentic reference material.

Preparation of [1, 6-^{14}C]1, 6-Hexamethylene bis (dicyandiamide)(4)(HMBDA).

[1, 6-^{14}C]1,6-Hexane diamine dihydrochloride (3)(452 mg, 2.4 mmol), sodium dicyanimide 95% (470 mg) and AR isopropanol pre-dried over molecular sieve (10 ml) was stirred and refluxed for 16 hours (bath temperature (90° - 100°C)), and heated for a further 20 hours at 85°C. The white suspension was centrifuged to separate and the product washed with water (3 x 6.0 ml) before drying under reduced pressure at 45°C for 16 hours, to give a white solid (339.5 mg; 56.8% Yield). This was all transferred to the next stage.

Preparation of 1, 1-[1, 6-^{14}C]Hexamethylene bis (5-(4 chlorophenyl) biguanide (5) (Chlorhexidine)

p-Chloroaniline (345 mg, 2.72 mmol) was stirred with AR isopropanol (1.2 ml), a solution of concentrated hydrochloric acid (252 mg) in AR isopropanol (1.4 ml) was added, and the mixture stirred for 30 minutes to give a clear solution. [^{14}C] HMBDA (4) (339.5 mg, 1.36 mmol) and AR isopropanol (3.0 ml) were introduced into the reaction mixture which was then stirred in a silicone bath at 85° - 90°C for 7 hours. After cooling the white gelatinous product was stirred at ambient temperature for 12 hours.

On addition of an aqueous sodium hydroxide solution (2.7%; 4.3 ml) the white dihydrochloride dissolved and the crude chlorhexidine base was slowly deposited (2 hours). The product was separated by centrifugation, and washed with isopropanol (2 x 1.0 ml), isopropanol-water (1:1) (2 x 1.0 ml) and water (4 x 1.0 ml) before being dried under reduced pressure at ambient temperature for 16 hours. This gave a white solid (509 mg; stage yield 74.2%). The overall yield of crude product based on potassium [^{14}C]cyanide was 25.2%.

The product was examined by TLC (System A) and was shown to be predominantly [^{14}C] chlorhexidine together with fourteen impurities. Autoradiography showed that all the components were radiolabelled. The different route of synthesis presented some additional difficulties in the purification procedures. An aliquot of the crude product was purified by the procedures described to yield product 1 and product 2. The remainder of the crude product was stored at 0-4°C.

PRODUCT 1

1) Chlorhexidine recrystallised readily from absolute ethyl alcohol. An aliquot of the crude [^{14}C] chlorhexidine was isotopically diluted (1:1) with unlabelled material and serially (10x) recrystallised from absolute ethyl alcohol to give an overall recrystallisation yield of 58%. At this point the radiochemical purity was 94%. The chromatographic pattern showed 4.5% of polar material retained at the origin. This was an unacceptable level of impurity.

2) The product (62.5 mg) was again isotopically diluted with an equal weight of pure unlabelled material and serially recrystallised a further five times. The product obtained, a white crystalline solid, was dissolved in the TLC solvent (System A) and applied to a small Merck Silica HR column (2.7 x 3.0 cm) which had been pre-washed with the developing solvent for 16 hours. Fractions (1.0 ml) were collected and a 5 µl aliquot of each was examined by TLC (System A).

The plates were then autoradiographed for 16 hours. Examination of the autoradiographs allowed selection of those fractions which contained a single component identical in R_f with pure chlorhexidine. These were combined and evaporated to dryness under reduced pressure from a water bath at 45°C to give a white crystalline solid (55.2 mg). Elemental analysis found C 52.1; H 6.0; N 26.3; $C_{22}H_{30}N_{10}Cl_2$ requires C 52.3; H 5.9; N 27.7.

TLC examination (System A) showed the product to be identical with pure reference chlorhexidine when viewed under UV 254 nm, and this was confirmed by autoradiography. Plate segmentation followed by scintillation counting gave radiochemical purities of 97.3% and 97.9%. Duplicate determinations using TLC system B gave radiochemical purities of 97.0% and 97.9%. The radiochemical purity using system C was 98.5%. Examination by gas-liquid chromatography-mass spectrometry (GCMS) produced the same fragmentation pattern as pure, reference chlorhexidine. A two-dimensional TLC using system A followed by autoradiography demonstrated that no decomposition or oxidation had occurred on the plate as a result of chromatographic examination.

The affinity of chlorhexidine for binding to glassware produced an unacceptable variability in specific activity determinations carried out by normal procedures. Acceptable reproducibility was achieved by preparing each solution of ^{14}C-labelled product in a methanol solution of unlabelled chlorhexidine (50% w/v). All glassware used in the determinations was also rinsed with the unlabelled chlorhexidine solution. Ten determinations gave a specific activity of 22.76 ± 0.43 μCi/mg [11.5 mCi/mmol]. An alternative method of specific activity determination by a combustion technique gave the specific activity as 22.3 μCi/mg and this confirmed the accuracy of the more conventional method.

PRODUCT 2

An attempt was made to obtain pure material by the above column method from the crude material in the recrystallisation liquors and the rejected fractions from the above column purification. These were combined and evaporated to dryness under reduced pressure. The residue was dissolved in the developing solvent (System A) and applied to a small silica HR column which had been pre-washed with the developing solvent. Fractions (1.0 ml) were collected and 2 µl aliquots of each fraction were applied to a silica GF plate and developed with the same system. The plates, when dried, were autoradiographed. In all fractions the impurity level was unacceptable.

The crude product was recovered from the fractions by evaporation under reduced pressure. The residue was dissolved in the minimum volume of the developing solvent and applied to 8 Merck Silica GF preparative plates which had been pre-washed in the same solvent. The plates on elution showed good separation into fourteen components. That these were all labelled with carbon-14 was confirmed by autoradiography. The band corresponding in R_f to the reference chlorhexidine was removed, formed into a small column and eluted with the developing solvent used (System A). When all the chlorhexidine had been removed, the eluate was evaporated to dryness under reduced pressure from a water bath at 30°C. The residue was dissolved in boiling absolute ethyl alcohol, centrifuged to separate traces of insoluble material, evaporated to half volume in a nitrogen stream and allowed to crystallise at 0° - 4°C. The white crystalline product, (177 mg) examined by TLC (System A), appeared to be homogeneous when viewed under UV 254 nm. Autoradiography, however, showed the presence of labelled impurities some of which were more polar and others less polar than chlorhexidine.

A small column of Silica GF (2.0 x 1.0 cm diameter) was formed and prewashed with the developing solvent for 16 hours. The [^{14}C] chlorhexidine was dissolved in the developing solvent and applied to the column and 1.0 ml

fractions were collected. TLC (System A) was used to examine each fraction (2 µl aliquot) and the plates autoradiographed. Fractions 15 - 50 were combined and evaporated to dryness under reduced pressure from a bath at 40°C. The residue was dissolved in the minimum volume of boiling absolute ethyl alcohol, centrifuged to separate a small amount of insoluble material and allowed to crystallise at 0° - 4°C for 4 hours. After separation by centrifugation, the product was washed with ice cold absolute ethyl alcohol (3 x 2.0 ml) before drying under reduced pressure at ambient temperature and yielded 137.6 mg of a white crystalline solid (last stage purification recovery 77.7%). The radiochemical purity of the product was found to be 98.8% (including slight tail) on System A and 96% on System B. The specific activity was determined as 10.25 ± 0.26 µCi/mg, (5.2 mCi/mmol).

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