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Research Article

Convenient synthesis of D-*threo*-[dichloroacetyl 1–^{14}C] chloramphenicol

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Summary
A convenient synthesis of chloramphenicol labelled with carbon-14 in the dichloroacetyl group at the 1 position is described. It was prepared as part of a 4-step sequence from [1-^{14}C] glycine and the product was purified by preparative HPLC. A radiochemical yield of 47% was obtained based on [1-^{14}C] glycine and the product had a specific activity of 0.47 mCi/mmol. The procedure can be employed for the synthesis of high specific activity [^{14}C] chloramphenicol, labelled at 1, 2 or both the positions of dichloroacetyl group. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: ethyl [1-^{14}C] diazoacetate; ethyl [1-^{14}C] dichloroacetate; D-*threo*-[dichloroacetyl 1-^{14}C] chloramphenicol

Introduction
The broad-spectrum antibiotic, chloramphenicol, was isolated from *Streptomyces venezuelae* in 1947.[1] It had a wide spectrum of activity only in its D-*threo* configuration against gram positive and gram negative bacteria, many anaerobic bacteria, chlamydia and Rickettsia.[2] It is widely used to treat typhoid, dysentery and bacterial infection of the eye. Although this drug was commonly used earlier, it is now reserved for microbial infections that are resistant to antibiotics that have fewer side effects. Chloramphenicol is primarily bacteriostatic. It binds to the 50S subunit of the 70S ribosomes and inhibits bacterial protein synthesis.

Quantitative measurement of transgenic expression is necessary to study the mechanism of eucaryotic genetic expression.[3,4] One way to study this is by use of the gene, expressing the enzyme – chloramphenicol acetyl transferase (CAT). When encoded by a bacterial drug-resistance gene, it inactivates chloramphenicol by acetylating the drug at one or both of its hydroxyl groups. This gene is

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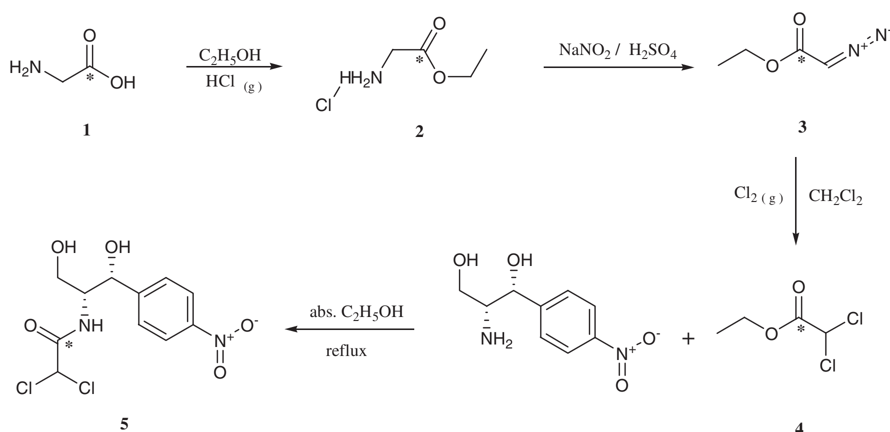
not found in eucaryotes and therefore eucaryotic cells contain little or no chloramphenicol acetyl transferase activity. Enzyme activity being low, its assay requires labelled chloramphenicol tagged with either carbon-14 or tritium.

Chloramphenicol acetyl transferase activity in transfected cells is monitored by liquid scintillation counting (LSC) or thin-layer chromatography (TLC).[5] Both LSC and TLC are performed in parallel from a single cell extract. Cell extracts are incubated in a reaction mixture containing ^{14}C- or ^{3}H-labelled chloramphenicol and *n*-butyryl coenzyme A. Chloramphenicol acetyl transferase transfers the *n*-butyryl moiety of the cofactor to chloramphenicol. After organic extraction, the radiolabelled butyryl chloramphenicol is then detected with either LSC or TLC followed by radiochromatogram scanning. Hence the need to synthesize the corresponding carbon-14 version of chloramphenicol arose. The preparation of chloramphenicol by condensing D-*threo*-2-amino-1-*p*-nitrophenyl-1,3-propanediol (chloramphenicol base) with methyl dichloroacetate is one of the several methods described in the literature.[6] Since the synthesis of chloramphenicol base is somewhat complex,[7–9] the labelled analogue prepared by Iliceto resulted in a very low radiochemical yield.[10] Carbon-14-labelled chloramphenicol, labelled in the dichloroacetyl portion of the molecule, was prepared by Ise[11] from malonic [2-^{14}C] acid with a radiochemical yield of 19.5%. Here, we present the preparation of D-*threo*-[dichloroacetyl 1-^{14}C] chloramphenicol **5** from [1-^{14}C] glycine **1** with the label situated in the dichloroacetyl group at the 1 position. The preparative procedure can very well be employed for the synthesis of high specific activity D-*threo*-[dichloroacetyl 1-^{14}C] chloramphenicol **5**, at 1, 2 or both the positions of the acetyl group. The procedure is efficient, easy to perform and the product obtained has a comparatively good radiochemical yield. The product was synthesized as part of a 4-stage sequence (Scheme 1).



Scheme 1.

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[1-^{14}C] glycine **1** on esterification was converted to [1-^{14}C] glycine ethyl ester hydrochloride[12] **2** which on diazotization formed ethyl [1-^{14}C] diazoacetate[13,14] **3**. Ethyl [1-^{14}C] diazoacetate **3** on chlorination was converted to ethyl [1-^{14}C] dichloroacetate[15] **4** which on condensation with chloramphenicol base gave D-*threo*-[dichloroacetyl 1-^{14}C] chloramphenicol **5**. In our cold synthesis on 2 mM scale, the reaction product was identified as chloramphenicol from its R_f value by comparison with the authentic sample. The pure product was obtained by preparative HPLC.[16,17] Further confirmation was obtained by determining the melting point along with the superimposable IR and UV spectra with an authentic sample of the product.

Experimental

The radioactive starting material, [1-^{14}C] glycine **1** was taken from ex-stock which was prepared in-house by another group following a published procedure.[18] Chloramphenicol and chloramphenicol base were procured from M/S Sigma. Other chemicals and solvents were obtained from M/S S.D. Fine Chemicals, India and were of A.R. grade. Melting point of the pure product obtained as well as the authentic sample of chloramphenicol was determined with an Expo capillary melting point apparatus. The radioactivity was determined using a LSS 4029 liquid scintillation counter supplied by Electronics Division, BARC, India. The radiolabelled compound was estimated using a Shimadzu UV-VIS spectrophotometer (Model UV – 265). TLC analysis was performed on a silica gel 60F (E. Merck) plastic sheet. The radiochromatograms were scanned with a Bioscan radiochromatogram scanner (Model AR 2000). HPLC analysis was performed using a Dionex HPLC system equipped with P680 HPLC pump and UVD 170U UV–detector. IR–spectra were recorded on a PYE UNICAM infrared spectrophotometer (Model SP3 – 300).

[1-^{14}C] *glycine ethyl ester hydrochloride* **2**

[1-^{14}C] glycine **1** (1.0 mCi, 2 mmol, 150 mg) and 10 ml of absolute ethanol were inserted in a long-necked Pyrex glass tube (15 mm OD and 20 cm long). Dry hydrogen chloride gas was gently bubbled through the solution while heating it at 65–70°C in an oil-bath. Gas flow was continued for 10 min more after the crystals dissolved in the solution. The solvent was removed *in vacuo* to give the title compound as a solid (0.98 mCi, 276 mg). Radiochemical yield: 98%.

Ethyl [1-^{14}C] diazoacetate **3**

Ethyl [1-^{14}C] diazoacetate **3** was prepared by using the specially designed reactor/extractor apparatus where the temperature was controlled by

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circulating cold water maintained at 10°C. The apparatus was specially fabricated so that the diazotization reaction and the subsequent extraction could be performed in the same apparatus. To a cooled mixture of (0.98 mCi, 1.98 mmol, 276 mg) [1-^{14}C] glycine ethyl ester hydrochloride **2**, 260 mg (3.7 mmol) of sodium nitrite, 8 mg (0.098 mmol) of sodium acetate, 5 ml of dichloromethane and 10 ml of water, taken in the reactor/extractor, and 100 μl of 10% aqueous sulphuric acid solution were added. The dichloromethane layer was thoroughly equilibrated, separated and passed with mild suction through a column (12 mm OD and 10 cm long) containing potassium carbonate which was maintained at the same reaction temperature. The aqueous layer in the reactor/extractor was subjected to diazotization which was repeated twice using 50 μl of 10% sulphuric acid each time. The total extract of dichloromethane (15 ml) obtained was used without purification for the next reaction.

Ethyl [1-^{14}C] dichloroacetate **4**

The combined dichloromethane extract of ethyl [1-^{14}C] diazoacetate **3** taken in a 100 ml R.B. flask was cooled in an ice-bath. Dry chlorine gas was gently bubbled through this solution until the yellow colour disappeared. Dichloromethane was removed by distillation and the product was dissolved in 5 ml absolute ethanol and assayed for radioactivity. 0.82 mCi (30.34 MBq) of the product obtained was used without purification for the next reaction.

D-*threo [dichloroacetyl 1–^{14}C]– chloramphenicol* **5**

A mixture of chloramphenicol base (424 mg, 2 mmol) and ethyl [1-^{14}C] dichloroacetate **4** (0.82 mCi) in absolute ethanol (6.5 ml) was refluxed for 6 h. The solution was allowed to cool to room temperature and ethanol was removed *in vacuo*. The solid residue was dissolved in ethyl acetate (30 ml) and washed with 1 N HCl (10 ml) followed by water (2 × 15 ml). The ethyl acetate layer was again washed with 5% $NaHCO_3$ (10 ml) followed by water (2 × 15 ml) and then dried over anhydrous sodium sulphate. On concentrating under reduced pressure, an oily residue resulted which was triturated with diethyl ether to give the title compound. The compound was purified by preparative HPLC using C-18 reverse-phase preparative column (25 cm × 2.5 cm) and methanol:water (37:63, v/v) as mobile phase at a flow rate of 4 ml/min. UV-detector was set at 278 nm (retention time for chloramphenicol = 69.43 min). The compound was then analyzed by silica gel TLC using the solvent system – chloroform:methanol (95:5). A single peak in the TLC radiochromatogram scan was obtained corresponding to the authentic sample. Radiochemical purity of the D-*threo*-[dichloroacetyl 1–^{14}C]–chloramphenicol **5** was estimated to be greater than 99%. The radiolabelled

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compound was estimated spectrophotometrically at 278 nm and radioactivity measured by liquid scintillation counter. The title compound (0.47 mCi, 0.99 mmol, 320 mg) was obtained with a radiochemical yield of 57.31%. Specific activity:0.47 mCi/mmol. The overall radiochemical yield of the title product based on [1-^{14}C] glycine **1** was 47%.

Results and discussion

The main problem we faced was the low yield of ethyl diazoacetate which subsequently led to the low yield of chloramphenicol. We used diethyl ether for the extraction of ethyl diazoacetate after the diazotization of glycine ethyl ester hydrochloride. Also the diazotization and extraction were carried out in two different apparatus. Ethyl diazoacetate is reported to decompose rapidly in the presence of acid. It is explosive and hence distillation even under reduced pressure is risky and dangerous. Therefore, the product should be used as soon as possible. Dichloromethane instead of diethyl ether protects the ester from the excess acid used in the diazotization reaction,[19] so we decided to use dichloromethane instead of diethyl ether for the extraction of ethyl diazoacetate and also for the next conversion to ethyl dichloroacetate. The apparatus was so designed that the temperature could be controlled by circulating cold water maintained at 10°C. It was fabricated so that the diazotization reaction and the subsequent extraction could be performed in the same apparatus. With the above modifications, we could successfully prepare labelled ethyl diazoacetate and subsequently chloramphenicol in good yield.

The prepared carbon–14-labelled chloramphenicol on analysis by silica gel TLC using the solvent system – chloroform:methanol (95:5) followed by autoradiography, showed three radioactive impurities along with radioactive chloramphenicol. Purification of this crude radiolabelled chloramphenicol was first tried by silica-gel column chromatography using the mobile phase containing chloroform:methanol (90:10) but separation was not achieved. Preparative HPLC purification was then tried following the analytical procedure as reported[16] using mobile phase 0.1 N sodium acetate and acetonitrile (85:15) in which the impurities merged with the product and no clear-cut separation was obtained as shown in Figure 1, whereas on using methanol:water (37:63) as mobile phase at a flow rate of 4 ml/min and UV-detector set at 278 nm and as followed by Zeegers and co-workers,[17] we successfully separated all the impurities and the radiochemically pure carbon–14-labelled chloramphenicol was obtained. It was observed that in the first HPLC purification process, the retention time for the impurities and chloramphenicol was found to be more or less close by whereas in the latter case good resolution was obtained as revealed by the vast difference in the retention time of the impurities and that of chloramphenicol which was found

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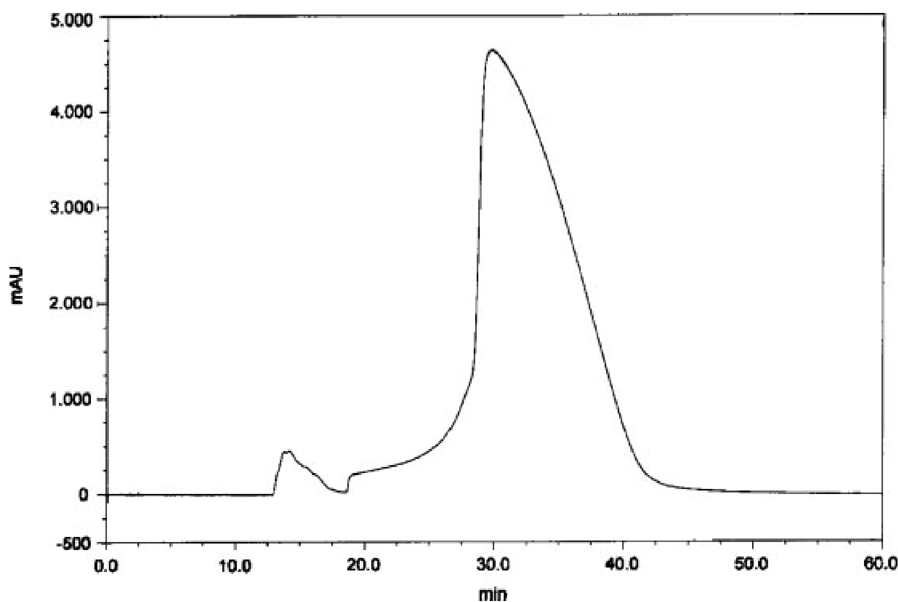

Figure 1. Preparative RP-HPLC chromatogram of the labelled chloramphenicol using solvent system: 0.1 N sodium acetate/acetonitrile (85:15)

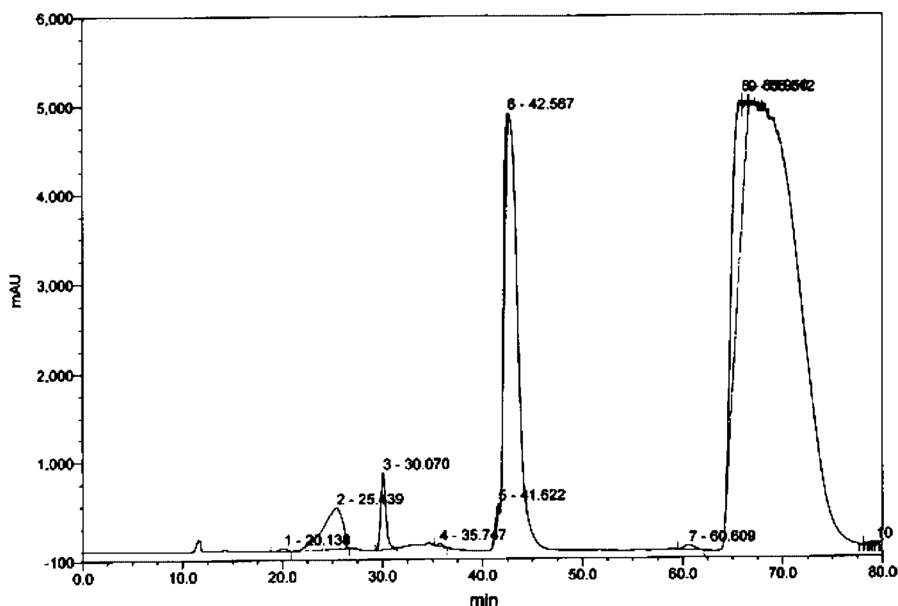

Figure 2. Preparative RP-HPLC chromatogram of the labelled chloramphenicol using solvent system: methanol/water (37:63)

to have got shifted to 69.43 min from 31 min as shown in Figure 2. Figure 3 shows RP-HPLC chromatogram of pure labelled chloramphenicol obtained on an analytical column.

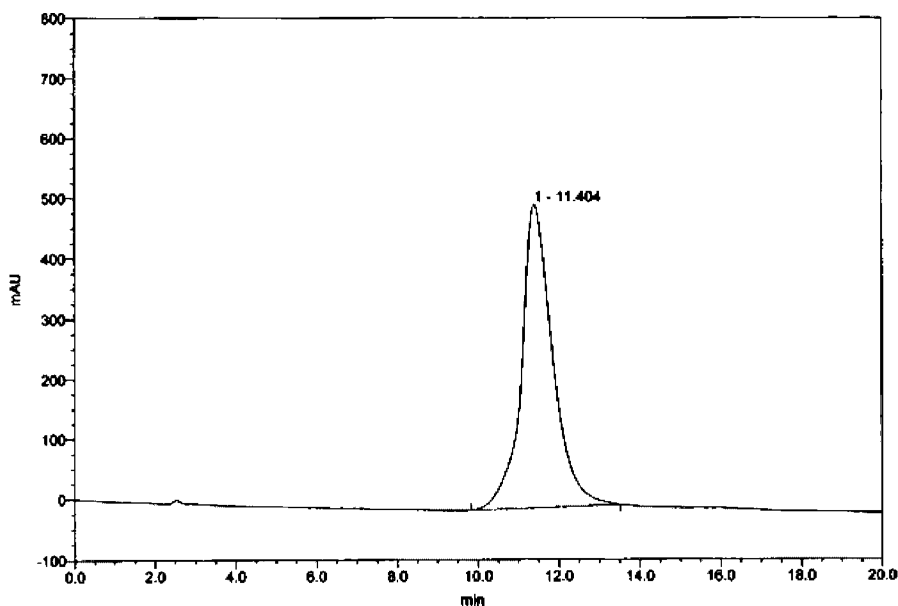

Figure 3. Analytical RP-HPLC chromatogram of the labelled chloramphenicol using solvent system: methanol/water (37:63)

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