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MICROSCALE SYNTHESIS OF HIGH SPECIFIC ACTIVITY ^{3}H-NITROGLYCERIN

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SUMMARY

The synthesis and purification of ^{3}H-nitroglycerin (glyceryl trinitrate, GTN) from commercially available ^{3}H-glycerol of high specific activity are described. The optimum microscale reaction conditions were defined. Purification of the product by thin-layer chromatography produced ^{3}H-GTN with an overall molar yield of 65% and a radiochemical purity of 99.0%.

Key Words: ^{3}H-nitroglycerin, organic nitrates, vasodilator drugs.

INTRODUCTION

Nitroglycerin (GTN) is an organic nitrate with potent vasodilator activity and is widely used in the management of several cardiovascular disorders (1). While this drug has been in use for over a century, the mechanism by which it induces relaxation of vascular smooth muscle is still poorly understood. Several hypotheses have been put forward to explain the action of GTN (2); one hypothesis proposes the existence of a specific receptor site for organic nitrates in smooth muscle cells (3). We proposed to test this hypothesis by using radioligand binding techniques common to the study of pharmacological receptors (4). For this reason radiolabelled GTN of high specific activity

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(10 Ci/mmol) and purity was necessary; unfortunately this was not commercially available, requiring its synthesis in the laboratory. Because of the high cost of the radiolabelled glycerol needed for ^{3}H-GTN synthesis, it was necessary to conduct this synthesis on a microscale. Although methods for moderate and large scale synthesis of organic nitrates are documented (5,6), no methods for the microscale synthesis of GTN that we required were found in the literature. We proposed to synthesize ^{3}H-GTN from glycerol by sulphuric acid catalyzed nitration of ^{3}H-glycerol. This involves the following two steps (7).

$$(1)\quad 2H_2SO_4 + HNO_3 \longrightarrow NO_2^+ + H_3^+O + 2HSO_4^-$$

$$(2)\quad NO_2^+ + R-OH \longrightarrow R-O-NO_2 + H^+$$

$$R = HOCH_2-CHOH-CH_2-$$

The chromatographic conditions used to obtain the high purity ^{3}H-GTN required for our experiments are also described.

EXPERIMENTAL

Materials

Uniformly labelled ^{3}H-glycerol (stated to be 38.2 Ci/mmol, radiochemical purity of 98.5%) was purchased from New England Nuclear-Dupont Inc. (Lachine, Quebec). All chemicals used in the synthesis were at least reagent grade, obtained from a variety of sources, and used without further purification. All solvents were of analytical grade and the diethyl ether was distilled over KOH pellets prior to use. Due to the interaction of GTN with medical plastics (8), the use of any plastic labware was avoided, and all glassware was silanized prior to use by treatment with trimethylchlorosilane (Pierce Chemicals, Rockford, IL). Water for all solutions was deionized with a Barnstead Nanopure® system. Thin-layer chromatography (TLC) was carried out on

Whatman K6 silica gel plates, 20 x 20 cm, 250 μm (Chromatographic Specialties Ltd., Brockville, Ontario). Radioactivity was measured with a Searle Mark III 6880 liquid scintillation system (Searle Analytic Inc., Des Plaines, IL) using Aquasol® (New England Nuclear-Dupont Inc., Boston, MA) as the scintillant. Minivials® were obtained from Fisher Scientific (Mississauga, Ontario) and the cone-shaped 5 ml Reactivial® was obtained from Chromatographic Specialties Ltd. (Brockville, Ontario). Gas-liquid chromatographic (GLC) analysis of GTN was carried out on a Hewlett-Packard 5730A gas chromatograph equipped with a ^{63}Ni electron capture detector (9).

Synthesis

As the ^3H-glycerol was supplied in a total volume of 50 ml ethanol and water (1/19, v/v), it was necessary to remove the solvent to attain anhydrous reaction conditions. This solution was divided into four equal volumes and these were lyophilized until the volume was reduced to approximately 3 ml. The concentrated glycerol solution was transferred to a 5 ml Reactivial®, that was the final reaction vessel. At this point, the ^3H-glycerol was diluted with an equal amount (0.25 mg, 2.7 μmol) of non-radiolabelled glycerol. This step was included in our protocol for economic reasons and to increase the quantity of starting material to the necessary 0.5 mg. Then the lyophilization was continued until all of the solvent was removed. Methanol (0.5 ml) was added and the solvent removed by lyophilization; this step was included to ensure that traces of water were removed (10).

Two hundred microlitres of a mixture of fuming HNO_3/H_2SO_4 (45/55, v/v) (10) were added to the vial and the contents were stirred briefly with a small glass rod. The vial was then capped and rotated so that the mixed acids wetted the sides of the vial and ensured complete mixing of the reactants. The reaction was allowed to proceed for 2 min at room temperature. To quench the reaction, 0.5 ml of ice-cold water was added to the reaction mixture and the vial was immediately placed in liquid nitrogen for 20-30 sec. The resulting mixture was then extracted with diethyl ether for 2 min (4 x 1 ml). To the pooled diethyl

ether extracts was added 1.5 ml of aqueous 0.6 M K_2CO_3 to neutralize the acids. After separation of the phases, the organic phase was dried by the addition of anhydrous $MgSO_4$. The organic phase was centrifuged at 100 xg for 10 min to pellet the $MgSO_4$ and the diethyl ether phase was saved. The $MgSO_4$ pellet was then washed extensively with diethyl ether and these wash volumes of diethyl ether were pooled together with the diethyl ether obtained after centrifugation. The pooled diethyl ether phase was dried under a stream of nitrogen to a volume of 200 µl.

Purification of GTN by TLC

The products of the synthesis were purified by TLC on a silica gel plate; the solvent system consisted of toluene and ethyl acetate (10/1, v/v) saturated with water. The diethyl ether phase containing the ^{3}H-GTN was applied to the plate as a 15 cm strip. A marker solution containing non-radiolabelled GTN, 1,2-glyceryl dinitrate (1,2-GDN) and 1,3-glyceryl dinitrate (1,3-GDN) was chromatographed on the same plate. The silica gel plate was allowed to develop such that the solvent front ran 15 cm from the origin. The area of the plate containing the products of synthesis was covered to protect it from the detection reagent. The Rf value of each of the organic nitrate compounds in the marker mixture was determined by spraying the uncovered portion of the plate with the detecting reagent (1% diphenylamine in methanol, w/v) and exposing it to UV light for 0.5 min. The GTN and GDN were then visible as brown spots. ^{3}H-GTN was obtained by scraping the silica gel from the TLC plate in the region corresponding to the Rf value for GTN and then extracting the silica gel scrapings with ice-cold acetone (4 x 2.5 ml). Identical TLC conditions were used to assess radiochemical purity of the synthesized ^{3}H-GTN. An aliquot of the acetone extract was chromatographed and individual bands 2-3 cm wide of the silica gel plate were scraped and placed in Minivials®, followed by the addition of 1.5 ml of deionized water and 5 ml of Aquasol®. Each vial was shaken to form a gel suspension and the radioactivity in each band was determined by liquid scintillation spectrometry.

Quantitation of GTN by GLC

To quantitate the yield of non-radiolabelled GTN during optimization of the synthetic procedure and to determine the specific activity of the radiolabelled GTN, GLC analysis was carried out under conditions similar to those described by Armstrong et al. (9). The chromatographic conditions were as follows: 6' (1.8 m) x 1/8" (3.2 mm) I.D. glass column packed with 10% OV-101 on Chromosorb 750®, 100/120 mesh; injection port temperature, 150°C; oven temperature, 130°C; detector temperature, 200°C; flow rate of argon/methane (95/5) carrier gas, 55 ml/min. With on-column injection, the retention times of GTN and the internal standard, dinitrobenzene, were 3.5 and 5.5 min respectively. The purified GTN was diluted 1/1,000 in benzene and 2 µl samples were injected onto the GC column. The content of GTN was quantitated by comparison of the peak height ratios with an appropriate standard curve.

RESULTS AND DISCUSSION

Due to the high cost of the ^{3}H-glycerol of the required specific activity, it was important to determine the reaction conditions and minimum quantity of glycerol required as starting material to produce a maximum yield of GTN. All experimental conditions were developed using non-radiolabelled glycerol. It was found that in order to drive the reaction to near completion, anhydrous reaction conditions were required; this included the use of fuming nitric acid. If less than 90% nitric acid was used, the reaction was partially quenched, and predominantly GDN was produced. Further, a 10-fold molar excess of nitric acid to glycerol was found to be the optimal ratio for maximal yield of GTN. The optimum reaction time was found to be 2 min, at room temperature; reaction times shorter (30 sec) and longer (10 min) produced less GTN. Initially the reaction conditions were tested by starting with 100 mg (1.09 mmol) and 10 mg (109 µmol) glycerol; both quantities gave consistent yields of 65-70%. The yield of GTN was similar with as little as 0.5 mg (5.4 µmol) of glycerol as

the starting material, but the yield was consistently decreased to 30% when the starting quantity was reduced to 0.25 mg (2.7 µmol). Thus, 0.5 mg (5.4 µmol) glycerol was the starting quantity in the radiolabelled synthesis made up of 0.25 mg of ^{3}H-glycerol and 0.25 mg unlabelled glycerol as described above.

The TLC conditions produced complete separation of GTN (Rf 0.50), 1,2-GDN (Rf 0.23) and 1,3-GDN (Rf 0.13) for the marker mix of non-radioactive compounds. By this method, the purity of the ^{3}H-GTN synthesized was assessed at 99.0% GTN with < 1% 1,2- and 1,3-GDN. The specific activity of the ^{3}H-GTN was 13.3 ± 1.3 Ci/mmol; this was lower than the specific activity anticipated (19.1 Ci/mmol). This may be attributed to the specific activity of the glycerol being less than stated on the label or the quantity received being less than 0.25 mg. The major product of the synthesis was verified to be GTN by (a) TLC, (b) GLC, and (c) bioassay. Another feature of this synthetic procedure is that it can be used to produce and purify high specific activity tritiated 1,2-GDN and 1,3-GDN; these may be important species in the investigation into the mechanism of vasodilation induced by GTN.

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