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The Preparation of Tritium-Labelled Ryanodine

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SUMMARY

A method for preparing tritium-labelled ryanodine is described. The alkaloid was first brominated, and the bromoryanodines were separated from the reaction mixture by thin layer chromatography; NMR analysis at this stage showed substitution of pyrrole protons by bromine. Bromoryanodine was first hydrogenated, and the product was purified and characterized as ryanodine, to establish the feasibility of the method. Tritium was then reacted with bromoryanodine to yield labelled ryanodine with a specific activity of 2.85 Ci/mmole. No detectable isotope exchange occurred when the labelled alkaloid was incubated in aqueous media at pH 7.0 and 30° C.

INTRODUCTION.

Ryanodine is a neutral alkaloid, obtained from the plant Ryania speciosa Vahl, which exerts profound effects on muscle, producing an irreversible contracture in skeletal muscle and a negative inotropic action in cardiac muscle [1]. The extreme potency of the drug and the apparent specificity of its effects make it a useful tool with which to study factors regulating muscle contraction and relaxation, but since there is no practical means of assaying ryanodine when present in small amounts in biological systems, the availability of a labelled form of the drug would be most desirable. Although the exact structure of ryanodine is not yet completely established, it is known to be an ester of pyrrole-2-carboxylic acid and a highly condensed polyhydric alcohol, ryanodol [2]. Selective re-esterification of ryanodol with a labelled acid is not possible; and labelling by the Wilzbach procedure [3] produces a mixture of materials which ultimately yields ryanodine with too low a specific activity to be of use in studies with subcellular muscle preparations [4]. An attempt was therefore made to introduce bromine into the pyrrole ring of ryanodine,

with the subsequent incorporation of tritium via a halogen replacement reaction [5]. This report describes the preparation by such means of labelled ryanodine of high specific activity.

EXPERIMENTAL.

Preparation of bromoryanodines.

Bromine water was added dropwise to a stirred aqueous solution of ryanodine (10 mg/ml) at room temperature until a faint yellow color persisted. The flocculent precipitate was filtered off, washed with a small volume of water and air-dried. The precipitate, consisting of the crude bromination product (CBP) was dissolved in methanol and examined by thin layer chromatography on Eastman Kodak #K301 R silica gel plates developed in methanol : chloroform (5 : 95). Four major components were present, having R_F values of 0.33, 0.53, 0.64 and 0.75. A few milligrams of each of these materials were separated on the TLC plates, eluted with methanol and hydrogenated as described below. After hydrogenation and subsequent chromatography it was found that only the material of R_F 0.53 yielded a hydrogenation product having the chromatographic characteristics of ryanodine when examined under conditions listed above and therefore no further attention was directed at the remaining components of CBP. Larger amounts of the 0.53 component were then obtained after chromatographing CBP on 2 mm preparative Brinkmann #HF 254/366 silica gel plates, with care being taken not to overload the plates; the extreme insolubility of CBP in other than polar solvents such as methanol or ethyl acetate precluded satisfactory separation by column chromatography. After eluting the 0.53 bands with methanol and removing the solvent a white crystalline material was obtained, referred to hereafter as bromoryanodine. Little success was achieved in numerous attempts to recrystallize bromoryanodine from various solvents, and the material was therefore examined at this stage of purity. The m.p. was 197-203° C, and analysis of bromine content indicated the presence of 2.9 atoms of bromine per molecule. Comparisons were made of the NMR spectra of solutions of ryanodine and of bromoryanodine in dimethylsulfoxide-d_6, with tetramethylsilane as internal reference; the spectra indicated that the bromoryanodine contained two components, the major one exhibiting the characteristic hydroxyl group signals seen with the parent ryanodine, but in addition having all the pyrrole ring C-H protons substituted by bromine, while the minor component had only two of the pyrrole protons substituted by bromine. Since satisfactory separation of these two components could not be obtained in any of the TLC systems examined, the mixture of bromoryanodines was then hydrogenated, after which the reaction products could be readily separated, as described below.

Bromine-hydrogen exchange.

The mixture of bromoryanodines (250 mg) was dissolved in a minimal volume (50 ml) of methanol then 50 mg each of 5 % palladium charcoal and of freshly prepared magnesium oxide were added, and the mixture was stirred under hydrogen at atmospheric pressure at 25° C for 30 minutes. The catalyst was filtered off, and the solvent was removed under vacuum; the solid residue was extracted three times with 10 ml chloroform, and the combined extracts were placed on a 2 × 30 cm column of silica gel suspended in chloroform. Elution was carried out with methanol : chloroform (5 : 95), with the eluate being monitored spectrophotometrically at 268 nm. Two peaks were obtained, accounting for approximately 90 % and 10 % of the total absorbance, and 133 mg of materials from the major peak component was obtained after removal of solvent and recrystallization twice from diethyl ether. The identity of this material as ryanodine was established by comparison with authentic ryanodine with respect to the following parameters : — melting points; I.R. spectra; U.V. spectra at pH 1 and 9; NMR spectra; chromatographic behaviour in solvent systems listed above and also in chloroform : methanol : acetic acid (75 : 20 : 5) and in methyl acetate : isopropanol : ammonia (45 : 35 : 20); biological activity as determined by LD_{50} in mice and also by inhibition of calcium transport in a skeletal muscle fraction [6].

Bromine-tritium exchange.

This was carried out at the Radiochemical Centre, Amersham, England, using scaled down but otherwise essentially similar conditions to those described above for the bromine-hydrogen exchange reaction; the rate of the exchange reaction was sufficiently high that little loss of tritium occurred via incorporation into the solvent under these conditions. Two radioactive compounds were obtained, corresponding to the major and minor components produced in the hydrogen exchange reaction described above. The major component was separated on TLC silica gel plates and rechromatographed in chloroform : methanol : acetic acid (75 : 20 : 5) and in methyl acetate : isopropanol : ammonia (45 : 35 : 20) until some 98 % radiochemical purity was attained, as determined from radiochromatogram scans. Examination of this material in all of the above solvent systems showed it to be indistinguishable from ryanodine, with a specific activity of 2.85 Ci/mmole.

A small amount of the labelled ryanodine was incubated for 2 hours at 30° C and pH 7.0 in the medium used for calcium uptake studies [6], followed by removal of the water by lyophilisation. No exchange of tritium into the medium could be detected under these conditions.

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