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Short Communication

Salbutamol radioimmunoassay: Synthesis and properties of the benzylic succinate of salbutamol

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Introduction

Salbutamol, which is used extensively in the treatment of asthma, is effective as a bronchodilator by its action on the β-2-adrenergic receptors of the bronchial and vascular smooth muscles. Tracer studies [1] have shown that after inhalation of tritiated salbutamol at doses of 84 and 220 μg, the peak plasma level averaged 0.95 ng ml^{-1} (n = 3) and 3.22 ng ml^{-1} (n = 4), respectively. In another experiment [2], where the tritiated drug was given separately in oral (8 mg) and in aerosol (0.08 mg) doses to patients, peak salbutamol plasma concentrations achieved were approximately 20 and 0.25 ng ml^{-1}, respectively.

The determination of salbutamol in biological fluids is normally achieved by the use of radio tracer methods in which tritiated salbutamol is administered together with unlabeled drug. Evans *et al.* [3] and Lin *et al.* [1, 2] have studied the metabolism of salbutamol in plasma and urine and have characterized the sulfate as one of the metabolites. Techniques such as countercurrent distribution, ion-exchange and thin layer chromatography were used in combination with liquid scintillation spectrometry. However, there have been few reports of non-radioisotopic assays for salbutamol. For instance, several GLC–MS methods using stable isotope standards with multiple ion recording in the electron impact mode [4–6] and in the chemical ionization mode [7–9] for plasma or serum drug level determination have been described. The sensitivity achieved with these GLC–MS assays is about 0.5–1.0 ng ml^{-1}. Recently, Oosterhuis and Boxtel [10] published an HPLC procedure with electrochemical detection for which a sensitivity of 0.5 ng ml^{-1} is claimed.

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The above methods suffer from a lack of sensitivity or specificity, tedious extraction procedures and/or derivatization techniques, and/or the use of expensive and sophisticated instrumentation as well as requiring standards of limited availability. Attempts to improve the sensitivity limits of some of the published methods such as GLC, GLC–MS and HPLC have not been successful. Also, since these methods are not readily amenable to routine clinical monitoring, a radioimmunoassay (RIA) approach was considered to offer advantages. RIA techniques are simple and sensitive, use a small volume of biological fluids and are readily applied to routine analysis. Synthesis of an appropriate salbutamol derivative has been undertaken and details of this work are now reported.

Experimental

Equipment

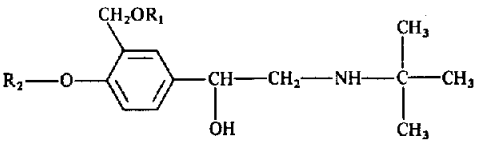
A Hewlett–Packard 5983A GLC–mass spectrometer (MS)-data system, operated in the electron impact mode with 70 eV as the ionization beam energy, and with a source temperature of 200°C, was used for MS analysis. The NMR spectra were recorded by means of a Bruker WR-80 Fourier transform spectrometer operated at 20.1 MHz for ^{13}C-NMR and at 80 MHz for ^{1}H-NMR at ambient temperature with tetramethylsilane (TMS) as internal standard. The IR spectra were recorded by means of a Beckman Microlab 620 MX computing infrared spectrophotometer at ambient temperature. All UV-VIS spectra were recorded on a Beckman DU-8B spectrophotometer maintained at constant temperature.

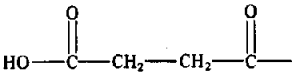
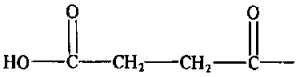
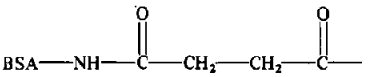
Preparation of free salbutamol

Salbutamol sulphate (1.0g) was weighed into a 100 ml Erlenmeyer flask and absolute ethanol (50 ml) added. The mixture was stirred mechanically and a mixed cation/anion exchanger (Rexyn I-300, H-OH, Fisher Scientific), equivalent to 5 ml volume, was added and stirring continued until the white suspension disappeared or until the resin became brown. If salt remained or the resin became brown, more resin was added. After 2 h stirring, the solution was filtered and the ethanolic filtrate evaporated to dryness under vacuum to yield a yellow oil. Ethyl acetate (100 ml) was added to yield a crop of white crystals. After standing overnight in a refrigerator, the solution was filtered and the crystals collected and dried. The crystals thus obtained had a melting point of 154–155°C with a yield of approximately 50% and represented free salbutamol (base) as confirmed by MS, IR, NMR, UV and TLC.

Synthesis of O-(3-carboxypropionyl) derivative of salbutamol (II)

Salbutamol (200 mg, 0.84 mmol) was dissolved in methanol (25 ml) and the solvent was removed under vacuum using a rotary evaporator. The yellow oily residue was redissolved in absolute ethanol (40 ml) using a magnetic stirrer. ^{14}C labelled succinic anhydride (114.5 mCi/mmol, New England Nuclear) in toluene was placed in a test tube and evaporated to dryness, then redissolved in ethanol (0.5 ml). While stirring, succinic anhydride (90 mg, 0.9 mmol), in slight molar excess, was added to the ethanol salbutamol solution together with the ethanolic tracer solution. After 5 min, all the cold succinic anhydride had dissolved and a cloudy white solution appeared. At the end of 2 h, the stirring was stopped, the solution was filtered, and the harvested crystals were washed with ethanol and dried. The crystals, obtained in a 60–70% yield, had a melting point of 143–144°C.



	R_1	R_2	Name
I	H	H	Salbutamol
II		H	Salbutamol (benzylic) succinate
III	H		Salbutamol (phenolic) succinate
IV		H	Salbutamol conjugate

Mass spectrometry gave diagnostic ions at *m/z* 223, 222, 221, 220, 209, 208, 190, 136, 135, 101, 100, 92, 91, 86, 74 and 45. IR (KBr) 3700, 2100 cm^{-1} (OH, COOH, NH); 1740 cm^{-1} (C=O, ester); 1715 cm^{-1} (C=O, acid) and 1620 cm^{-1} (aromatic). UV spectrum in 0.1 M NaOH gave two maxima at 244 and 295 nm with molar absorptivity values of 11 000 and 3200 respectively. The ^{13}C-NMR at 20.1 MHz in deuteriated acetic acid gave 178.7, 172.0, 156.4, 132.5, 129.2, 128.8, 123.8, 117.2, 70.6, 63.5, 59.1, 49.1, 30.0 and 29.7. The ^{1}H-NMR at 80 MHz in deuteriated acetic acid or methanol gave 1.45 ppm (s, $C(CH_3)_3$), 2.68 and 2.71 ppm (d, CH_2–CH_2), 3.34 ppm (m, CH_2–N), 5.2 ppm (m, CH–OH and CH_2–O) and 7.0 ppm (m, aromatic).

Coupling of salbutamol succinate to bovine serum albumin (IV)

The mixed "cold/tracer" salbutamol succinate (40 mg, 0.12 mmol) was dissolved in a solution of dioxane (25 ml), distilled water (3 ml) and triethylamine (300 µl). The colourless solution was stirred for 30 min at room temperature and isobutyl chloroformate 30 µl (0.23 mmol) added and the stirring continued for 2 h. This solution was then added dropwise to a bovine serum albumin (BSA) solution (86 mg, 0.00112 mmol in 25 ml distilled water) contained in a 100-ml round bottom flask. The resultant colourless solution was stirred overnight at room temperature. The mixture was then dialysed (Fisher Scientific dialyser tubing) against distilled water containing 0.2% m/v sodium azide for four days with occasional changes (three times on first day, then once daily). Following lyophilization, the conjugate was further purified by passing through a Sephadex column (G. Fine). Protein fractions were collected, combined, counted and concentrated with an Amicon concentrator using a 25 mm 25 PM 10 Drafco membrane. The final product, estimated to contain not more than 38 hapten residues per mole conjugate (n = 38), was lyophilized.

Immunization

The hapten–protein conjugate was dissolved in normal saline and emulsified with complete Freund's adjuvant. The conjugate was administered to four New Zealand male

white rabbits by injection of 0.5 ml of the emulsion into each flank. At 2-week intervals thereafter, the injections were repeated with the emulsion prepared as described except that complete Freund's adjuvant was replaced by incomplete adjuvant.

Serum was obtained from the marginal ear vein at bi-monthly intervals after the third injection. The antiserum titer of each rabbit was checked by evaluating the binding characteristic of tritiated salbutamol (17.1 Ci/mmol; Nuclear Research Center, Negev, Israel) to each antiserum. After optimal titers were achieved, blood was obtained from each rabbit by cardiac puncture. The harvested serum in each case was immediately lyophilized and stored at −70°C.

Results and Discussion

The major problem encountered during the work was the solubility of salbutamol. The first attempt to synthesize salbutamol succinate involved use of the yellow oil (see preparation of free salbutamol), redissolving it with ethanol then treating with succinic anhydride. This approach was successful. In order to eliminate possible impurities in the oil, it was found necessary to recrystallize the salbutamol from ethyl acetate. The synthesis has been repeated many times and has been found to be reproducible. It should be noted that the use of solvents other than ethanol to dissolve the oil prior to reaction leads to crystallization of free salbutamol, thus inhibiting the esterification reaction.

The UV spectra for salbutamol and salbutamol benzylic succinate were recorded at 37°C over the pH range 4.0–9.0. In basic medium, both compounds gave similar molar absorptivities for the maxima at 244 nm (≈11 000) and 295 nm (≈3200). At pH 5.1, in a 0.02 M sodium acetate buffer, maxima were observed at 220 and 276 nm for salbutamol and its succinate, respectively, with molar absorptivities of 8800 and 2137. Salbutamol succinate was found to be stable in basic medium for at least 9 h, thereafter a third maximum was observed at 335 nm which increased with time, indicative of some form of degradation.

At one stage, radioactive ^{14}C succinic anhydride was used for the synthesis of a mixed "cold/tracer" salbutamol benzylic succinate. The reaction was performed using 100 mg of salbutamol with the amounts of ethanol and succinic anhydride in the same ratio as outlined in the Experimental section. Radioactive succinic anhydride in toluene (130 μl) was added along with the "cold" succinic anhydride. The crystals obtained had a melting point of 169–172°C which differs from those of salbutamol and salbutamol benzylic succinate. No reasonable explanation other than the presence of traces of toluene or possibly the ^{14}C succinic anhydride could be given. The synthesis was repeated without the radioactive chemical but 130 μl of toluene was added. The crystals thus obtained once again had a melting point of 169–172°C. The spectroscopic characteristics of the product suggest that succinate derivatization occurs at the phenolic position (III). It is recommended that the toluene be evaporated and the radioactive succinic anhydride redissolved with ethanol prior to reaction.

The antiserum was evaluated by incubating with salbutamol in 200 μl of pH 7.4 buffer. Following a charcoal separation technique, the "percentage bound" was determined. The resulting logit versus log of concentration curve was linear over the 0.05–2.00 ng range with a correlation coefficient of 0.99 ($n = 20$). Preliminary evaluation indicated that the curve is not sensitive to plasma volume over the 50–500 μl range.

A radioimmunoassay sensitive down to the low picogram range was developed for salbutamol. The assay does not appear to be influenced by plasma. Full method

validation would require an extensive metabolic study of salbutamol in humans. Several authors have reported the use of combined HPLC–RIA to investigate drug metabolism [11]. This approach with other spectroscopic methods will allow the investigators to determine the specificity of the RIA method.

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