PREPARATIVE ISOLATION OF [U-^{14}C]SOLANESOL FROM $^{14}CO_2$-CHAMBER GROWN TOBACCO

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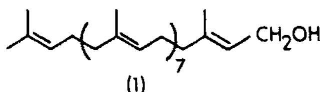
ABSTRACT

A method for the preparative isolation of [U-^{14}C]solanesol from $^{14}CO_2$-chamber grown tobacco is described. Freeze-dried tobacco leaves were Soxhlet extracted with methylene chloride. Fractionation of the extract by silica gel chromatography yielded crude solanesol. Subsequent purification by normal phase high pressure liquid chromatography yielded [U-^{14}C]solanesol with a total activity of 474 µCi, a specific activity of 0.5 mCi/mmol, and a radiochemical purity of 95% as determined by RP-HPLC. The chemical purity was 97% and the chemical identity of the isolated compound was confirmed by co-chromatography with reference material and by mass spectroscopy.

KEY WORDS: tobacco, [U-^{14}C]solanesol, preparative HPLC, radiochromatography.

INTRODUCTION

Solanesol (1) a trisesquiterpene alcohol, was first isolated from tobacco in 1956[1] and its structure reinvestigated and revised in 1959[2] to (2E, 6E, 10E, 14E, 18E, 22E, 26E, 30E)- 3,7,11,15,19,23,27,31,35-nonamethylhexatriaconta-2,6,10,14,18,22,26,30,34-nonaen-1-ol. It is one of the major



(1)

isoprenoids of tobacco with the highest levels accumulating in the chloroplasts[3]. One focus of interest has been on elucidating the contribution, direct or indirect, of solanesol to the flavor and aroma of tobacco smoke[4]. In studying the chemistry of precursor-product relationships in a burning cigarette an important tool is the use of carbon-14 labelled compounds[5]. Since such studies generally require ^{14}C-labelled compounds of high radiochemical purity, it was of interest to develop methodology to isolate and analyze uniformly radiolabelled solanesol from $^{14}CO_2$-chamber grown tobacco.

In early studies of the biosynthesis of lipids in tobacco, Reid[6] reported the isolation and purification of ^{14}C-labelled solanesol from tobacco by

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repetitive chromatography on alumina. The purity of the isolated ^{14}C-solanesol was checked by comparison of its infrared spectrum with authentic solanesol. Generally, with reference to more recent methods for isolating unlabelled solanesol, the compound is extracted with hexane[7] or methylene chloride[8]. The extraction is followed by further fractionation by column chromatography on silica[7,9] or florisil[4]. However, solanesol purified by column chromatography, recrystallization or thin layer chromatography (TLC) remains contaminated by closely related isoprenoids like solanesenes and bombiprenone[7]. Preparative high-pressure liquid chromatography (HPLC) results in further purification, as reported by Severson et al.[7], although minor unidentified impurities are still observed.

Various methods for assaying solanesol qualitatively and/or quantitatively have been reported: infrared spectrophotometry[7,10], thin layer chromatography and densitometry[11,12], gas chromatography (GC)[7-9] and HPLC[12-15]. Of these methods, GC, TLC and HPLC are amenable to on-line analyses for radiochemical and chemical purity. However, GC analysis of solanesol necessarily involves prederivatization or hydrogenation since direct GC analysis of solanesol yields thermal dehydration products[7]. The observed thermal instability of solanesol makes TLC and HPLC more attractive methods for micro-analysis and/or micro-isolation of ^{14}C-solanesol.

This paper describes the preparative isolation of 95% radiochemically pure [U-^{14}C]solanesol using a combination of liquid chromatography techniques.

RESULTS AND DISCUSSION

The labelled tobacco was produced by growing the plants in a $^{14}CO_2$ growth chamber as described by Bass et al.[16] Growing conditions were similar to those previously shown to yield uniformly labelled carbon-14 plant material[17]. For this reason the isolated solanesol was assumed to be also uniformly labelled. Freeze-dried leaves from labelled tobacco plants were extracted with methylene chloride in a Soxhlet apparatus. Assay of the radioactive extract by HPLC indicated approximately 750 mg of solanesol to be present, about 0.7% by weight

of the freeze-dried leaves. Components eluting earlier than solanesol were observed both by refractive index and UV (254 nm) detection. Solanesol shows no selective absorption at wavelengths >220 nm.

Flash chromatography fractionation of the crude extract on silica gel according to polarity yielded a major solanesol-containing fraction (1.4g, 1.2 mCi). This fraction was further purified by column chromatography on Kieselgel 60, to give two solanesol-containing fractions: Fr A (175 mg, 167 µCi) and Fr B (613 mg, 571 µCi). Both fractions were then purified by preparative HPLC on a Whatman Magnum 20 Partisil 10 column. All chromatograms showed two major features: (1) The major peak measured by refractive index was that of solanesol and (2) the major impurities were UV-absorbing and eluted earlier than solanesol. The chromatograms of Fr A showed relatively greater amounts of the early eluting components than Fr B, confirming the results obtained by TLC. Minor peaks were observed close to the solanesol peak and as far as practicable, the center of the solanesol peak was collected as the purified solanesol fraction. The combined fractions yielded solanesol having a total activity of 474 µCi (517 mg, 0.5 mCi/mmol), representing a radioactivity recovery of 64% based on the total radioactivity of Fr A and B. The yield based on the total weight of Fr A and B was 65%.

Identification of the HPLC-purified material as solanesol was confirmed by mass spectroscopy (m/e 631, M^{+}+1, m/e 612, M^{+}-H_2O). Qualitative thin-layer chromatography on silica gel of the ^{14}C-solanesol in 4 different TLC solvent systems indicated one homogeneous spot, with R_f values in all systems identical with authentic solanesol.

Thin-layer radiochromatography (TLRC) on silica gel of the ^{14}C-solanesol showed one major radioactive peak with 97 to 98% of radioactivity co-chromatographing with reference solanesol (R_f 0.59). A minor radioactive peak representing 1 to 2% of the total radioactivity was evident at R_f 0.4 to R_f 0.5. Radioanalysis of a mixture of ^{14}C-solanesol and authentic carrier solanesol gave similar results for radioactivity distribution. Visualization of the

chromatograms with 5% anisaldehyde spray showed the ^{14}C-sample to consist of one major spot (solanesol, R_f 0.59) and two minor spots at R_f 0.41 and 0.46 (corresponding to the minor radiochemical impurities). The mixture of ^{14}C-solanesol and carrier solanesol showed only one spot, R_f 0.59, the concentration of the minor spots being too dilute to be detected. This confirmed that the minor impurities observed in the ^{14}C-sample (and in the reference sample) are real impurities and not artifacts arising from decomposition of the solanesol during spotting or development. The chromatogram of reference solanesol showed, apart from the major spot at R_f 0.59, minor impurities at R_f 0.77 and R_f 0.9.

Reversed-phase high performance liquid radiochromatography (RP-HPLRC) analyses of the ^{14}C-solanesol were performed using a Waters Radial-Pak™ C-18

TABLE 1

Radiochemical Purity Determination by HPLC of [U-^{14}C]solanesol

Run #*	Retention time and % of Radioactivity	Refractive Index (Att 8X) Peak Retention time
1	22.4 min, >99% 10 min, trace	22.4 min (weak)
2	22.4 min, 95.9% 15 min, 0.4% 10 min, 3.8%	22.4 min (strong) 10 to 15 min (weak)
3	22.4 min 94.1% 10.2 min, 3.9% 11 to 14 min, 1.3% 14 to 16 min, 0.6%	22.4 min (strong) 10-16 min (weak) 29 min (weak)
4	(Reference solanesol)	22.4 min (strong) 38 min (weak)
5	22.4 min, 95.7% 10 min, 4.3%	22.4 min (strong)

* Run #1: 5 µl ^{14}C-sample (19 µg, 4.5 nCi)
#2: 50 µl ^{14}C-sample (190 µg, 45 nCi)
#3: 100 µl ^{14}C-sample (380 µg, 90 nCi)
#4: 100 µl reference carrier (340 µg)
#5: 70 µl admixture of ^{14}C-sample and carrier (about 340 µg total solanesol)
(Flow rate 1.25 mL/min)

cartridge with methanol as the eluting solvent. On-line radioactivity monitoring was done with a Berthold Model LB504 HPLC radioactivity monitor. The results are summarized in Table 1. Samples were simultaneously monitored for radioactivity, refractive index (RI) and UV absorption at 254 nm and 365 nm. Comparison of retention times for the ^{14}C-samples and carrier solanesol, as well as co-injection with carrier solanesol, clearly identified the major radioactive peak at 22.4 min to be solanesol. Both reference solanesol and ^{14}C-solanesol exhibited weak UV-absorbing impurities (254 nm) co-eluting partially with solanesol. In the case of the ^{14}C sample the early eluting impurities were also radioactive. Co-injection of ^{14}C-sample and carrier solanesol showed a composite of UV-absorptions of both ^{14}C and reference samples, confirming that these impurities were real and not artifacts arising during chromatography. Based on runs 2, 3 and 5 (Table 1) the average radiochemical purity of the HPLC-purified ^{14}C-solanesol was 95%. Comparison of the HPLC and TLC data showed the former to be a more sensitive method for quantitating the radioactive impurities.

In a separate series of runs, simultaneous determinations of radiochemical and chemical purities by the RP-HPLRC method were carried out. The results for the chemical purity for the ^{14}C-solanesol sample are summarized in Table 2.

TABLE 2

Chemical Purity Determinations by RP-HPLC of [U-^{14}C] Solanesol*

Run #	Refractive Index R.T. (min)	Area %
1 (25 µl, 220 µg)	13.0	>99.9
2 (25 µl, 220 µg)	13.0	98.5
3 (40 µl, 350 µg)	13.1	97.8

* Flow rate: 2.0 mL/min. Reference solanesol elutes at 13 min, with weak RI signals at 6 and 9 min, and weak UV signals at 13,14 and 19 min.

Purified ^{14}C-solanesol was kept below 0°C in hexane since there are reports in the literature on the decomposition of purified solanesol on standing at ambient temperature[1,6]. Prior to purification all crude fractions of ^{14}C-solanesol were also kept refrigerated, again because of published reports on the formation of insoluble resin on standing[13b] or when exposed to light and air[6]. Some increase in viscosity was observed for crude solanesol Fr A and B over a 4-day period. Comparison of the radiochemical purities of ^{14}C-solanesol determined 2 and 7 weeks after HPLC purification were 95 and 94% respectively. This suggests that little or no decomposition had occurred over this period. In contrast, a sample of ^{14}C-solanesol that had been stored at 3-4°C in acetonitrile/methanol/methylene chloride about 6 weeks showed a radiochemical purity of 92%, as determined by TLRC on silica gel. The radiochemical impurities were evident in the radiochromatogram. Chemical visualization of the chromatogram with 5% anisaldehyde spray showed a number of minor spots closely corresponding in R_f to the radiochemical impurities all with lower R_f on silica than solanesol.

In conclusion, preparative HPLC was found to be an effective method for purifying ^{14}C-solanesol from tobacco leaves. Radiochemical and chemical purities were effectively determined by RP-HPLC, with TLC serving as a useful complementary technique.

EXPERIMENTAL

General: All solvents were HPLC-grade (either Baker or Burdick and Jackson). Hexane and ethyl acetate (EtOAc) were redistilled. Column chromatography was performed on Kieselgel 60 (230-400 mesh, E. Merck). Stock solanesol was obtained from Sigma Chemical Co. High pressure liquid chromatography (HPLC) analyses were carried out using a Waters M-6000 pump with a U6K injector. Preparative isolation of ^{14}C-solanesol by HPLC was carried out using a Waters M-6000 pump with an extended flow range unit. Waters Model 401 refractometer was used for solanesol detection and Waters Model 440 absorbance detector monitoring at 254 nm and 365 nm for detection of UV absorbing impurities. Radiochemical purity was determined on-line with a Berthold Model LB504

HPLC Radioactivity Monitor. Chemical purity was determined by on-line integration of the refractometer output using a Hewlett-Packard Model 3390 reporting integrator. Thin layer chromatography (TLC) was carried out on 0.25 mm thickness silica gel 60 F-254 plates from E. Merck. The chromatograms were visualized either by charring with 70% sulphuric acid spray, spraying with 5% anisaldehyde (ANS) spray reagent (in acidified 95% ethanol) or with 3.5% molybdatophosphoric acid spray (MPA) reagent (EM Reagents). Solanesol gives a blue spot with MPA. With ANS a series of color changes is observed finally to yield a violet/blue color. Since R_f values are dependent on the activity of the plates, authentic solanesol was co-chromatographed with ^{14}C-samples for each TLC plate. Thin layer radiochromatography (TLRC) was performed using a Berthold Model LB282 TLC Linear Analyzer.

A. Extraction from ^{14}C-Tobacco Leaves

98.6 g of freeze-dried ^{14}C-tobacco leaves SC 58 were extracted in a Soxhlet apparatus in three portions. Each portion was extracted with 1 liter of methylene chloride for about 23 hours. The combined Soxhlet extracts, after evaporation of solvents under reduced pressure yielded 6.4 g of a green oil. Based on an external calibration table using authentic solanesol as reference, normal phase HPLC assay indicated about 750 mg of solanesol to be present. Micro-collection of the solanesol peak followed by liquid scintillation counting confirmed it to be radioactive. Radioactivity of the entire green oil was estimated at 8 mCi.

B. Flash Chromatography Fractionation

The green oil was redissolved in 250 mL hexane and fractionated by flash chromatography on 270 g Kieselgel 60 in 2 portions. In Run #1, about 125 mL of the crude material was layered onto the silica gel and eluted with (1) 500 mL hexane (2) 400 mL hexane/benzene (3:1) (3) 420 mL ether (4) 420 mL ether (5) 500 mL methanol. In Run #2, the remainder of the crude extract was chromatographed, with minor modifications in the eluting solvents. Some hexane-insoluble material (1.2 g, 0.8 mCi) remained in the crude extract flask and was not chromatographed. The layered material was eluted with (1) 500 mL

of hexane (2) 300 mL of hexane/benzene (1:2), (3) 300 mL of benzene/ether (1:2), plus 100 mL ether (4) 350 mL of ether (5) 500 mL of MeOH (6) 500 mL of CH_2Cl_2. Fractions of the same polarity from each run were combined. TLC analysis (10 X 20 cm plate, hexane/EtOAc, 4:1, reference solanesol R_f 0.4) indicated Fraction 4 (eluted with ether) to be the major solanesol-containing fraction. After removal of solvents under reduced pressure, this fraction yielded a green oil having a total activity of 1.25 mCi and weighing 1.48g.

C. Column Chromatography of Crude Solanesol

A 45 cm X 2.5 cm I.D. glass column was slurry packed with 50 g of Kieselgel 60 in hexane. The crude solanesol was layered onto the column as a solution in hexane and eluted initially with 120 mL of hexane, followed by 100 mL each of mixtures of hexane and ethyl acetate: (a) 95:5 (b) 90:10 (c) 75:25, twice (d) 60:40. Fractions were analyzed by TLC. Two solanesol-containing fractions were obtained: Fr A (20 mL) and Fr B (40 mL) (eluted with 3:1 hexane/EtOAc). By TLC Fr A was less pure than Fr B. After concentration under reduced pressure and removal of traces of solvent under vacuum (0.1 mm Hg) oily yellow residues were obtained from Fr A (167 μCi, 175 mg) and Fr B (571 μCi, 613 mg).

D. Preparative Normal Phase HPLC of Solanesol Fractions A and B

High resolution semipreparative HPLC purification of Fr A and B was performed on a Whatman Magnum 20 Partisil 10 column (50 cm X 2.2 cm I.D.). The samples were injected as solutions in hexane. Fr B was purified in six portions (70-100 mg each) and Fr A in 4 portions. The eluting solvent was 10% ethyl acetate in hexane for Run #1 (flow rate 15 mL/min), 15% ethyl acetate in hexane for all subsequent runs, at flow rates of 9.9 mL/min for Run #3 and 15 mL/min for all other runs. The solanesol containing fractions were combined, concentrated under reduced pressure and transferred quantitatively with hexane to a tared vial. Excess solvent was then evaporated under a stream of nitrogen and the resulting yellow oil was dried under vacuum (0.1 mm Hg) to constant weight to yield a cream-colored solid, 517 mg, total radioactivity 474 μCi.

Solanesol yield based on combined weight of Fr A and B (788 mg, 738 μCi) was 65%, the radioactivity yield being 64%. The percent yield based on the weight of freeze-dried leaves was 0.5%.

E. Reversed-Phase High Pressure Liquid Radiochromatography Analysis of [U-^{14}C] Solanesol

The HPLC-purified ^{14}C-solanesol was analyzed by reversed-phase HPLRC using a Waters Radial-Pak™ C-18 cartridge, with methanol as eluting solvent. Flow rates of 1.25 mL/min and 2.0 mL/min were utilized in separate runs. Results are summarized in Table 1 (Results and Discussion Section). The average radiochemical purity for the purified ^{14}C-solanesol was 95%. The chemical purity was determined to be ca. 97%. (See Table 2).

F. TLC of ^{14}C-Solanesol

(a) Eight 5 X 20 cm TLC plates were each spotted with 5 μl (19 μg) of ^{14}C-solanesol solution and 5 μl (50 μg) of reference sample. Duplicate plates were developed in each of four different solvent systems. One chromatogram of each pair was then visualized with MPA spray reagent, the other with ANS spray. In all instances, one homogeneous spot was observed for ^{14}C-solanesol with an R_f identical with that obtained for reference solanesol. Solvent systems and R_f values: (1) Hexane, R_f 0.0; (2) Hexane: 1,2-dichloroethane:acetone, 18:2:1, R_f0.19; (3) Chloroform, R_f0.36 (4) Chloroform/ethyl acetate, 9:1, R_f 0.73.

(b) The following solutions were prepared and analyzed on one 20 X 20 cm TLC plate. For each sample 5 μl of the solution was spotted (about 50 μg).

(1) Reference solution, 9.7 mg/mL CH_2Cl_2

(2) ^{14}C-solanesol, 10 mg/mL CH_2Cl_2 (about 9 nCi/μL)

(3) Admixture of 0.1 mL each of ^{14}C-solution and reference solanesol (approximate concentration of total solanesol 9.75 mg/mL with radioactivity concentration 4.4 nCi/μl).

The plate was developed in $CHCl_3$/EtOAc (27:3) and then analyzed for radioactivity distribution on a Berthold Model LB 282 TLC linear analyzer. 97 to 98% of the total radioactivity of ^{14}C-solanesol was found to be associated with one peak co-chromatographing with reference solanesol (R_f 0.59). Visualization of the chromatograms was done with ANS spray.

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