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PURIFICATION OF RADIOLABELED PHARMACEUTICALS

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SUMMARY

The chromatographic purification of radiolabeled compounds of diverse structural types, in milligram to gram amounts, is described. The compounds purified were a steroid, a D-1 receptor antagonist, an ACE inhibitor and a small peptide mimic. The purification methods used included isocratic normal-phase high-performance liquid chromatography (HPLC), gradient reversed-phase HPLC, counter-current chromatography and the use of a normal-phase gravity column with fraction monitoring by rapid analytical HPLC.

INTRODUCTION

Radiolabeled compounds are essential tools in modern drug research and development[1] and are particularly valuable in drug metabolism studies[2]. Radiolabeled compounds must be of high radiochemical and chemical purity[3] in order to conduct meaningful experiments. High purity is most effectively achieved using liquid chromatography. Pharmaceutical radiochemistry groups are called on to provide structurally diverse compounds, at high purity, in milligram to gram amounts. The rational development of efficient purification methods, using basic chromatography theory[4], is described below.

The steroid[^{3}H]mometasone furoate (**1a**) was purified by isocratic normal-phase high-performance liquid chromatography (HPLC). A solvent system was developed using thin-layer chromatography (TLC). The optimum column loading was established using analytical HPLC. The developed system was used on a semi-preparative column to purify 25-mg amounts of [^{3}H]mometasone furoate.

[^{3}H]Sch 39166 (**3**), a D-1 receptor antagonist, was purified by gradient reversed-phase HPLC. Using analytical HPLC, the capacity factor (k') and peak shape were optimized and a feasible loading was established. The separation was transferred to a semi-preparative column. Gradient elution allowed the loading of the relatively insoluble sample in a large solution volume with sample enrichment at the column head and subsequent elution of the compound in small volume.

[^{14}C]Sch 34826 (**4**) is a small peptide mimic which was purified on a gram scale using a gravity column packed with 12–25 μm silica gel. The separation was first run on a small column and scaled up by increasing the column diameter; all other parameters were held constant. Rapid analytical HPLC with both UV and radioactivity detection was used for fraction analysis.

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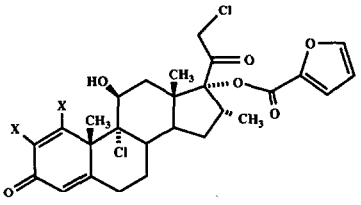
The ACE inhibitor [^{14}C]spiraprilat (**5**) is difficult to purify by column chromatographic methods because of its poor solubility properties and impurity profile. The compound was succesfully purified by counter-current chromatography on a coil planet centrifuge instrument. A suitable solvent system was chosen based on partition coefficients in two-phase systems of varying polarities and pH. [^{14}C]Spiraprilat was purified in 150–200-mg amounts using a 300-ml column.



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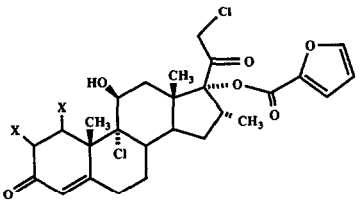

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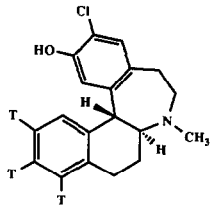

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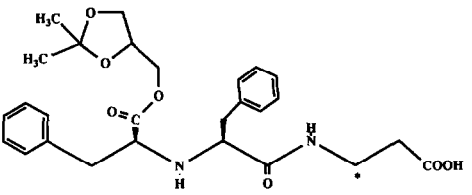

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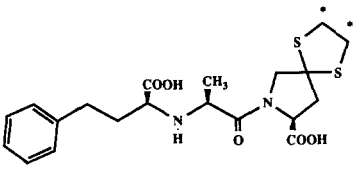

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EXPERIMENTAL

*[^{3}H]Mometasone furoate (**1a**)*

The HPLC instrument consisted of a Model 6000A pump and a U6K injector (Waters Assoc., Milford, MA, U.S.A.), a 970A variable-wavelength UV–VIS detector (Tracor, Austin, TX, U.S.A.) set at 254 nm and a Recordall Series 5000 recorder (Fisher Scientific, Springfield, NJ, U.S.A.) and Model LB-5025 radioactivity detector (Berthold Analytical, Nashua, NH, U.S.A.).

Analytical reversed-phase HPLC was carried out on a 25 cm x 4.6 mm I.D. LC-8 column (Supelco, Bellefonte, PA, U.S.A.). The mobile phase was methanol–water (35:65) at a flow-rate of 1 ml/min. Aquassure liquid scintillation cocktail (DuPont/NEN, Boston, MA, U.S.A.) at a flow-rate of 2 ml/min was used in the radioactivity detector. Normal-phase chromatography was carried out on 25 cm × 4.6 mm I.D. and 50 cm × 9.4 mm I.D. Partisil 10 silica columns (Whatman, Clifton, NJ,

U.S.A.). The mobile phase was dichloromethane–methyl *tert.*-butyl ether (93:7) at flow-rates of 1 and 4 ml/min, respectively.

[^{3}H]Sch 39166 (3)

The preparative HPLC system consisted of an LC-85 UV spectrophotometer set at 280 nm (Perkin-Elmer, Norwalk, CT, U.S.A.), Model 6000A and 510 pumps and a Maxima 820 chromatography workstation (Waters Assoc.), a Model 7125 injector with a 1.0-ml loop (Rheodyne, Cotati, CA, U.S.A.) and an M9 Partisil 10 CCS/C_8 column (50 cm × 9.4 mm I.D.) (Whatman). The flow-rate was 4.0 ml/min.

The analytical HPLC system consisted of a Model 490 multiwavelength spectrophotometer set at 280 nm, a Model 600 multi-solvent delivery system, a Model 840 data and chromatography control station, a Model 712 WISP autoinjector (Waters Assoc.), a Flo-One Model CT radiochemical detector with a 500-μl flow cell, (Radiomatic, Tampa, FL, U.S.A. and a RAC II Partisil 5 CCS/C_8 column (10 cm × 4.6 mm I.D.) (Whatman). The mobile phase consisted of methanol (solvent A) and 0.1 *M* ammonium acetate adjusted to pH 5 with glacial acetic acid (solvent B). The flow-rate was 1.0 ml/min and the temperature was ambient. Aquassure liquid scintillation cocktail was used for analytical radioactivity flow monitoring at 2.0 ml/min. The isocratic mobile phase was A–B (50:50). The gradient program was as follows: segment 1, A–B (50:50) isocratic for 10 min; segment 2, A–B (50:50) to 100% A linear gradient over 5 min; segment 3, 100% A isocratic for 15 min.

[^{14}C]Sch 34826 (4)

Preparative chromatography was carried out in glass Chromaflex columns (Kontes, Vineland, NJ, U.S.A.). The columns were packed with 12–25-μm LPS-1 silica gel (Whatman), slurried in chloroform. The slurry was briefly sonicated before packing. Compounds were placed on the columns as chloroform solutions and eluted with chloroform–methanol–glacial acetic acid (100:3:0.5). Fractions were collected in a Frac 100 fraction collector (Pharmacia, Piscataway, NJ, U.S.A.) and analyzed by HPLC. The HPLC system consisted of a Model 8800 gradient controller, Model 834 automatic sampler, Model 870 pump and column compartment equipped with a 50-μl loop Valco A45 injector (DuPont, Wilmington, DE, U.S.A.), Model 490 programmable multi-wavelength detector (Waters Assoc.) set at 220 nm and Model LB 5025 radioactivity detector (Berthold Analytical). Data reduction was done on a Model 840 data and chromatography control station (Waters Assoc.). A 10 cm × 4.6 mm I.D. Partisil 5 RAC II ODS-3 column was used with a mobile phase composed of methanol–acetonitrile–0.05 *M* K_2HPO_4 (pH 7.4) (20:20:60) at 1 ml/min. Aquassure liquid scintillation cocktail at a flow-rate of 1.5 ml/min was used in the radioactivity detector.

[^{14}C]Spiraprilat (5)

Counter-current chromatography was carried out on an Ito Model 1 multilayer coil separator–extractor equipped with a 300-ml coil (P.C. Inc., Potomac, MD, U.S.A.), a Model 6000A HPLC pump (Waters Assoc.) and a Model 7125 injector with a 2-ml loop (Rheodyne). Fractions were collected by a Frac 100 (Pharmacia) fraction collector. The solvent system was chloroform–methanol–2% acetic acid at a flow-rate of 4 ml/min.

Fractions were analyzed by HPLC. The HPLC system consisted of a Model 490 multi-wavelength spectrophotometer set at 220 nm, a Model 600 multi-solvent delivery system, a 712 WISP autoinjector, a Model 840 data and chromatography control station (Waters Assoc.) and a Flo-One Model CT (Radiomatic) with a 500-μl flow cell. The column was a 5-μm Partisil 5 RAC III ODS-3 (10 cm x 4.6 mm I.D.) (Whatman). The liquid scintillation cocktail was Aquassure at a flow-rate of 1.5 ml/min. The mobile phase was methanol–acetonitrile–0.05 *M* K_2HPO_4 (pH 7.5) (20:20:75) at 1.0 ml/min.

RESULTS AND DISCUSSION

*[^{3}H]Mometasone furoate (**1a**)*

[^{3}H]Mometasone furoate (**1a**), after attempted purification by preparative TLC and recrystallization, contained the 1,2-dihydro derivative **2a** as the major contaminant. Compound **2a** co-chromatographed with an authentic sample of **2b** prepared by an unambiguous route and characterized spectroscopically. A radiochromatogram of **1a** is shown in Fig. 1.

Mometasone and its dihydro derivative **2a** were barely resolved by reversed-phase HPLC. Additionally, a 125-mg batch required purification. A normal-phase HPLC system giving adequate separation of the two components ($\alpha \geqslant 1.2$) and acceptable loading (25 mg per run) was needed. A synthetic mixture of **1b** and **2b** (97:3) in dichloromethane (20 mg/ml) was used to develop a preparative HPLC sys-

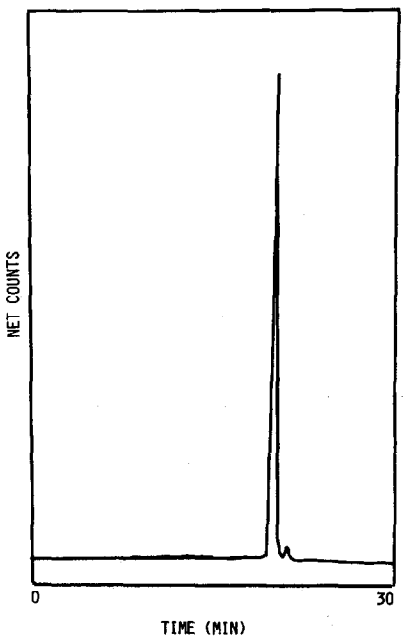


Fig. 1. Analytical reversed-phase radiochromatogram of crude [^{3}H]mometasone furoate (**1a, 2a**). Column, Supelco LC-8 (25 cm × 4.6 mm I.D.); mobile phase, methanol–water (35:65) at 1.0 ml/min; Aquassure liquid scintillation cocktail at 2.0 ml/min.

TABLE I

COLUMN LOADING RESULTS FOR A 25 cm × 4.6 mm I.D. COLUMN

Mass injected	*k'*	
	*Sch 32088 (**1a**)*	*Dideutero-Sch 32088 (**2a**)*
100 μg	5.7	4.4
500 μg	5.7	4.3
750 μg	5.6	4.4
1.0 mg	5.4	4.3
1.5 mg	5.6	4.3
2.0 mg	5.6	4.3
2.5 mg	5.7	4.4

tem. Solvent systems were screened on silica gel TLC plates. The solvent mixture dichloromethane–methyl *tert.*-butyl ether (93:7) was chosen because adequate separation was achieved ($\alpha = 1.3$), the calculated retention ($k' = 6$) on a column was acceptable (approximate k' values were calculated from TLC mobility data using the relationship $k' = (1 - R_F)/R_F$ from ref. 5, the solvents were volatile and readily removed and **1a** was very soluble in this mixture. A loading study on a 25 cm × 4.6 mm I.D. silica gel column was carried out with the test mixture (Table I).

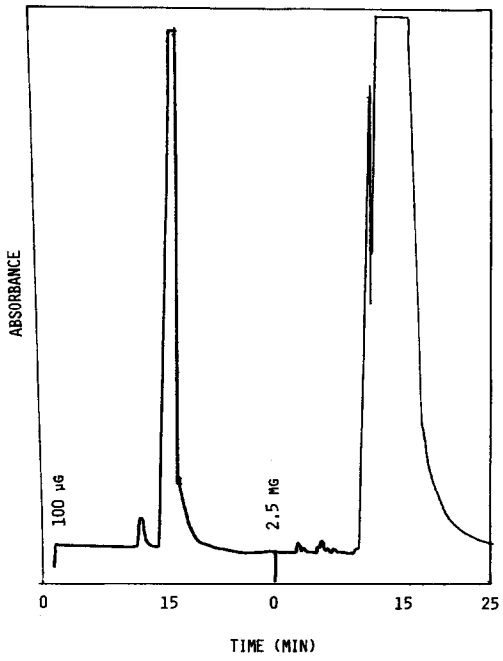


Fig. 2. Loading study of **1b–2b** synthetic test mixture. Column, Whatman Partisil 10 silica (25 cm × 4.6 mm I.D.); mobile phase dichloromethane–methyl *tert.*-butyl ether (93:7) at 1.0 ml/min.

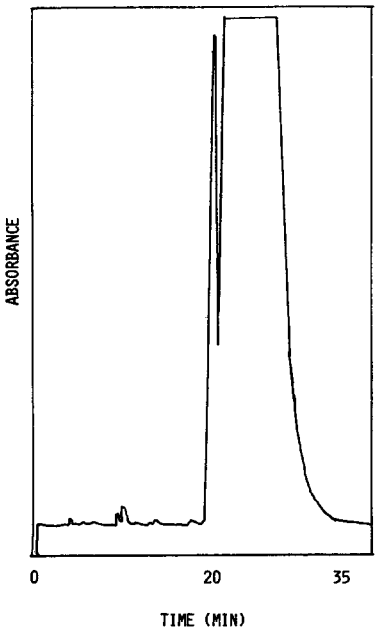


Fig. 3. Injection of 25 mg of **1a–1b** test mixture. Column, Whatman Partisil 10 silica Magnum 9 (50 cm × 9.4 mm I.D.); mobile phase, dichloromethane–methyl *tert.*-butyl ether (93:7) at 4.0 ml/min.

No change in k' was noted for a 2.5-mg compared with a 0.1-mg injection (Figure 2), although the bands had broadened. Thus the column had been loaded up to its "loading limit" for touching-band separation[6]. The separation was transferred directly to a 50 cm × 9.4 mm I.D. semi-preparative column and injections of 5 and 25 mg of the test mixture were made. The results, shown in Fig. 3, indicate that a 25-mg loading was acceptable, even though k' was slightly decreased.

[^{3}H]Mometasone furoate was purified in 25-mg portions. A typical preparative chromatogram is shown in Fig. 4. The yield of pure compound was 88% with a radiochemical purity of 98.9%. A radiochromatogram of the purified compound is shown in Fig. 5.

[^{3}H]Sch 39166 (3)

[^{3}H]Sch 39166 was prepared by platinum catalyzed exchange in tritiated water–trifluoroacetic acid[7] (labeling chemistry was carried out at the National Tritium Labeling Facility, Lawrence Berkeley Laboratory, U.C. Berkeley, Berkeley, CA, U.S.A.).

A radiochromatogram of the crude exchange product is shown in Fig. 6. Major radioactive impurities were tritiated water (11%) and strongly retained materials (4%) eluted when the reversed-phase column was stripped with methanol.

For purification of [^{3}H]Sch 39166, a 50 cm × 9.4 mm I.D. reversed-phase column was used in a gradient mode. This allowed the sparingly soluble [^{3}H]Sch 39166 to be loaded in a large volume with enrichment at the column head and subsequent elution in a small volume.

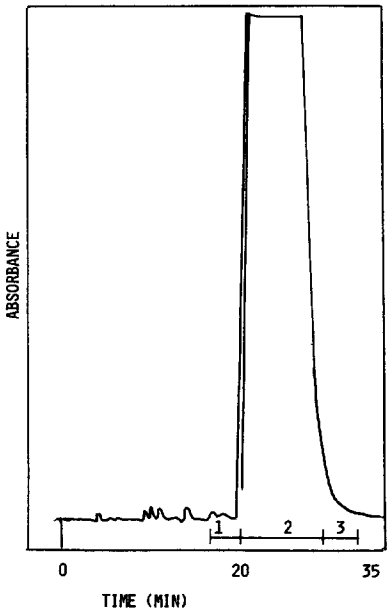


Fig. 4. Injection of 25 mg of crude [^{3}H]mometasone furoate (**1a, 2a**). Column, Whatman Partisil 10 silica Magnum 9 (50 cm × 9.4 mm I.D.); mobile phase, dichloromethane–methyl *tert.*-butyl ether (93:7) at 4.0 ml/min.

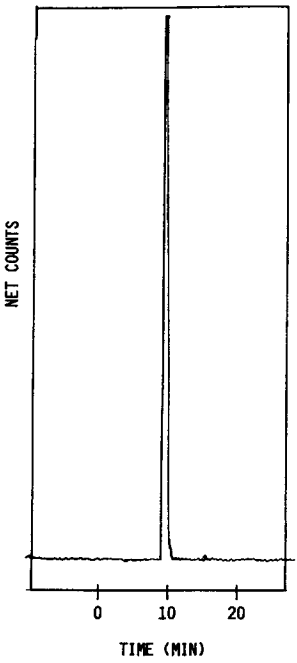


Fig. 5. Analytical reversed-phase radiochromatogram of purified [^{3}H]mometasone furoate (**1a**). Column, Supelco LC-8 (25 cm × 4.6 mm I.D.); mobile phase methanol–water (35:65) at 1.0 ml/min; Aquassure liquid scintillation cocktail at 2.0 ml/min.

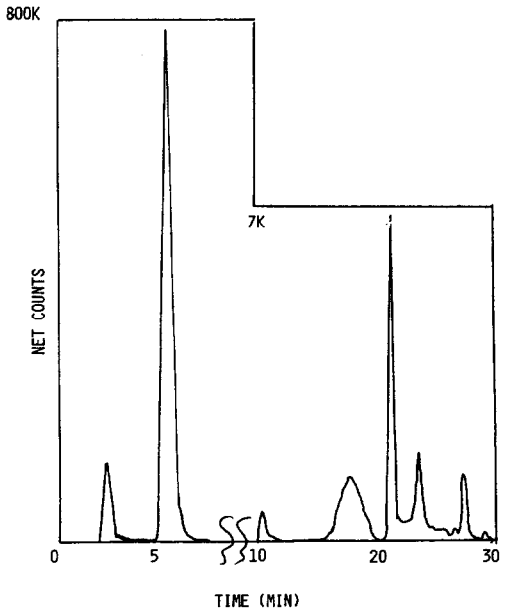


Fig. 6. Composite analytical radiochromatogram of crude [^{3}H]Sch 39166 (**3**). Column, Whatman Partisil 5 CCS/C_8 RAC II (10 cm × 4.6 mm I.D.); mobile phase, methanol–0.1 *M* ammonium acetate at 1.0 ml/min; 10-min isocratic (50:50) separation of tritiated water from [^{3}H]Sch 39166; detector sensitivity was then increased for the detection of strongly retained impurities during a 5-min linear gradient to 100% methanol and a 15-min isocratic column strip.

The column was equilibrated in 100% buffer at 4 ml/min. A 400-ml volume of sample was loaded into the injector loop and injected onto the column, followed by a 1-min (4-ml) buffer rinse. The process was repeated a further five times for a total sample load of 3.8 mg in 2.4 ml of methanol. A 15-min linear gradient to methanol followed by a 15-min isocratic elution was then run. Fractions were collected as shown on the preparative chromatogram in Fig. 7. Fractions 3 and 4 are [^{3}H]Sch

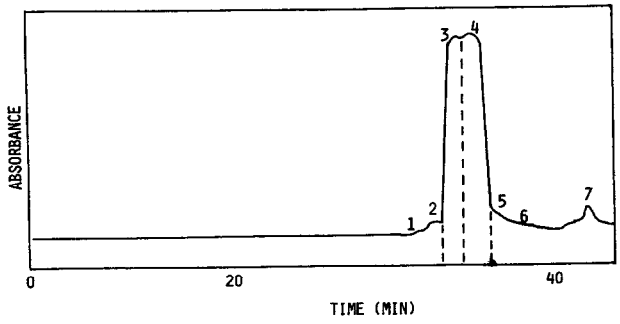


Fig. 7. Semi-preparative column injection of crude [^{3}H]Sch 39166 (**3**). Column, Whatman Partisil 10 CCS/C_8 Magnum 9 (50 cm × 9.4 mm I.D.); mobile phase, (1) 0.1 *M* ammonium acetate isocratic for 15 min (2) 15-min linear gradient to methanol, (3) 15-min methanol isocratic; flow-rate, 4.0 ml/min.

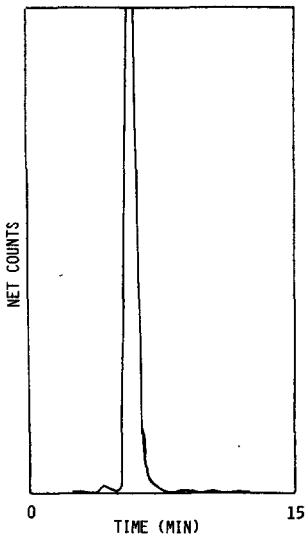


Fig. 8. Analytical reversed-phase radiochromatogram of purified [^{3}H]Sch 39166 (3). Column, Whatman Partisil 5 CCS/C_8 RAC II (10 cm × 4.6 mm I.D.); mobile phase, methanol–0.1 *M* ammonium acetate (50:50) at 1.0 ml/min; Aquassure liquid scintillation cocktail at 2.0 ml/min.

39166; cut-points are indicated by dotted lines in Fig. 7. These fractions represent a 94.7% recovery of the [^{3}H]Sch 39166 radioactivity applied to the column. Fractions were combined based on rapid analytical HPLC with radiochemical flow monitoring. After purification and pooling of all the tritium-exchanged reaction batches, a final batch purity of 97.5% was obtained. Fig. 8 shows a representative [A–B (50:50) isocratic] analytical radiochromatogram.

[^{3}H]Sch 34826 (4)

Chromatographic columns used for the purification of radioactive materials frequently become contaminated and cannot be used for other projects. Therefore, expensive preparative HPLC columns capable of handling gram amounts of material are rarely used in radiochemical purifications. Gravity liquid chromatography is a viable alternative. Crude [^{14}C]Sch 34826 contained several chemical and radiochemical impurities, as shown in Fig. 9. A 212-mg portion was purified on a 30 cm × 2.5 cm I.D. silica gel column using the solvent system chloroform–methanol–glacial acetic acid (100:3:0.5). Silica gel with a small particle size (12–25 μm) was slurried in chloroform and briefly sonicated, then poured into the column. One column volume of chloroform was passed through the column to stabilize the bed. The compound was dissolved in 1 ml of chloroform, carefully applied to the column and eluted with mobile phase. Fractions were collected and those with significant radioactivity were examined by HPLC. Pure fractions were pooled and evaporated. The purification was scaled up by chromatographing 925 mg of crude [^{14}C]Sch 38426 on a 30 cm × 4.8 cm I.D. column. The preparative chromatogram is shown in Fig. 10. HPLC analyses of the fractions are shown in Table I. It can be seen that purity across the main peak in the preparative chromatogram was not uniform. Fractions were com-

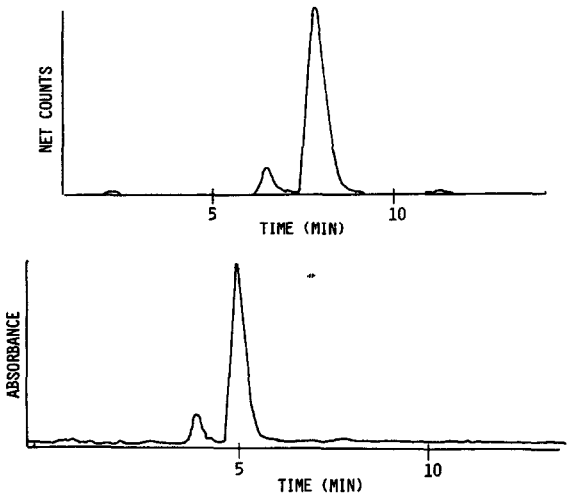


Fig. 9. Analytical UV (bottom) and radiochromatograms (top) of crude [^{14}C]Sch 34826 (**4**). Column Whatman Partisil 5 ODS-3 RAC II (10 cm × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.05 *M* K_2HPO_4 (pH 7.4) (20:20:60) at 1.0 ml/min; Aquassure liquid scintillation cocktail at 1.5 ml/min.

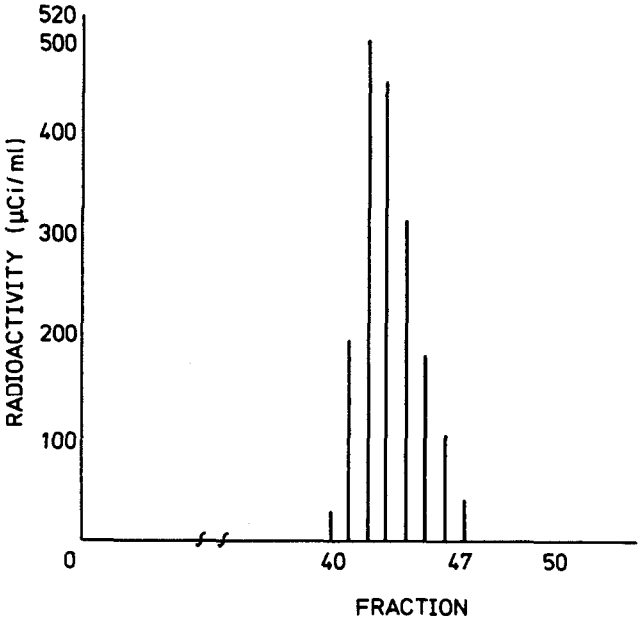


Fig. 10. Bar graph of liquid scintillation counting results for [^{14}C] 34826 (**4**) gravity column fraction aliquots. Column, 30 cm × 4.8 cm, packed with 12–25-μm Whatman LPS-1 silica gel; mobile phase, chloroform–methanol–glacial acetic acid (100:3:0.5).

TABLE II

REVERSED-PHASE HPLC FRACTION ANALYSIS

Fraction	*Radioactivity (μCi/ml)*	*Sch 34826*	
		Radiochemical purity (%)	*Chemical purity (%)*
40	33	90.8	90.9
41	203	98.5	99.0
42	512	100	100
43	470	97.7	97.3
44	324	95.7	94.2
45	192	94.8	93.5
46	111	94.3	93.1
47	39	93.8	92.9

bined based on analytical HPLC data. The yield of applied radioactivity in pure fractions from both columns was 64%.

A radiochromatogram of the purified material is shown in Fig. 11. Difficult separations may be effected by gravity LC by using silica gel of small particle size and careful fraction monitoring by rapid HPLC analysis.

[^{14}C]Spiraprilat (5)

[^{14}C]Spiraprilat has previously been purified by recrystallization or gravity LC with only marginal success; the highest radiochemical purity achieved was 96%. The poor solubility of spiraprilat in most solvents inhibited the development of alternative purification methods such as ion-exchange chromatography. Counter-current chromatography[8] proved to be an excellent method for purifying this compound. Potential solvent systems were chosen by literature analogy[9].A partition coefficient of 0.2–5

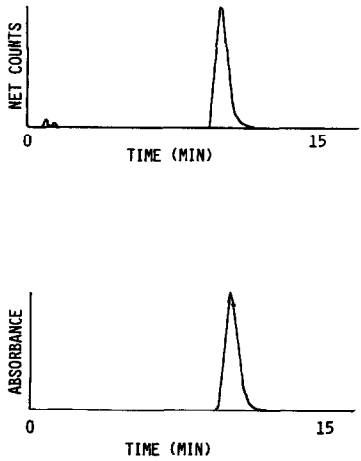


Fig. 11. Analytical UV (bottom) and radiochromatograms (top) of purified [^{14}C]Sch 34826 (**4**). Column, Whatman Partisil 5 ODS-3 RAC II (10 cm × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.05 *M* K_2HPO_4 (pH 7.4) (20:20:60) at 1.0 ml/min; Aquassure liquid scintillation cocktail at 1.5 ml/min.

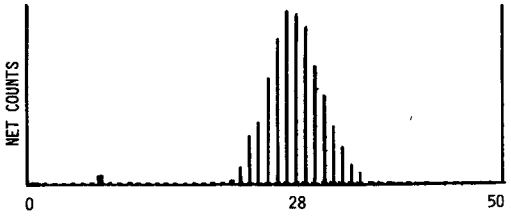
TABLE III

SOLVENT SYSTEMS FOR COUNTER-CURRENT CHROMATOGRAPHY

Solvent system	*Partition coefficient*	*pH of upper phase*
Methanol–chloroform–2% acetic acid (1:1:1)	1.5	3.3
Methanol–chloroform–water (1:1:1)	0.14	4.5

is recommended[10]. Crude [^{14}C]Spiraprilat was partitioned between equal volumes of upper and lower phases of the solvent systems shown in Table III. The relative radioactivity in both layers was determined by liquid scintillation counting. As can be seen in Table III, manipulation of partition coefficients by manipulation of pH is very effective for an ionizable compound such as spiraprilat. The solvent system chloroform–methanol–2% acetic acid (1:1:1) was chosen because of its desirable partition coefficient and its ability to dissolve large amounts (50 mg/ml) of spiraprilat.

The 300-ml coil was first loaded with stationary upper phase and the sample [70 mg in 1.5 ml of methanol–chloroform–2% acetic acid (1:1:1)] was injected. The mobile lower phase was pumped through the coil at 4 ml/min and 8-ml fractions were collected. An aliquot of each fraction was mixed with liquid scintillation cocktail for radioactivity measurements. Fig. 12 shows a typical histogram. Fractions 24–34 were pooled and the solvent was removed.



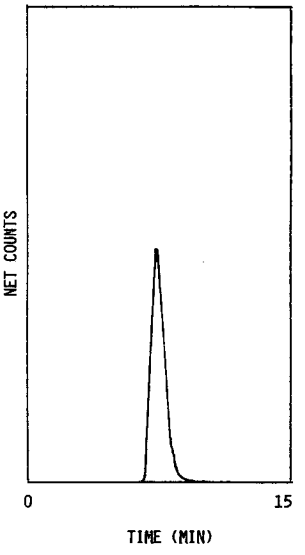


Fig. 12. Histogram of liquid scintillation counting analysis of [^{14}C]Spiraprilat (**5**) preparative counter-current chromatographic fraction aliquots. Ito multi-layer coil separator–extractor, 300-ml coil; mobile phase, chloroform–methanol–2% acetic acid (1:1:1) at 4.0 ml/min.

Fig. 13. Analytical reversed-phase radiochromatogram of purified [^{14}C]Spiraprilat (**5**). Column, Whatman Partisil 5 ODS-3 RAC II (10 cm × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.05 *M* K_2HPO (pH 7.5) (20:20:75) at 1.0 ml/min; Aquassure liquid scintillation cocktail at 1.5 ml/min.

Purification of 300 mg of crude reaction product was performed in several 70-mg runs, with an overall 67% mass recovery. Analytical HPLC with radioactivity flow monitoring gave a final batch radiochemical purity of 99.0% (Fig. 13). This is a significant increase over the best value (96%) previously achieved by recrystallization.

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