HIGH-EFFICIENCY PREPARATIVE-SCALE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF 14C-LABELLED ANTIBIOTICS

DONALD J. MORECOMBE

Radiochemistry Unit, Scientific Services, Beecham Pharmaceuticals Research Division, Medicinal Research Centre, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD (U.K.)

SUMMARY

The 14C-labelled antibiotics [2-14C]mupirocin, and [thienyl-3-14C]temocillin cannot be satisfactorily purified on a small scale by conventional methods of chromatography or recrystallisation. Their purification was successfully achieved by high-efficiency preparative-scale reversed-phase high-performance liquid chromatography. The purifications employed 250 mm × 10 mm I.D. or 22 mm I.D. stainless-steel columns packed with Merck LiChrosorb RP-18 (10 μm) stationary phase which were eluted with aqueous buffer solutions at flow-rates of 10–25 ml min^{-1} using conventional analytical instrumentation.

INTRODUCTION

In recent years the use of preparative-scale high-performance liquid chromatography (HPLC) has become increasingly widespread and is now regarded as a powerful technique for the purification of organic/bio-organic compounds[1-3].

We have found that the technique is particularly suitable for the purification of valuable radiolabelled pharmaceutical intermediates and products, where often the prime requirements are for rapid and good recovery of product at high purity on a moderate scale (< 500 mg). We have also used the technique to repurify existing stock materials that have undergone decomposition on storage.

Several commercially available preparative-scale HPLC systems employ mechanically compressed columns containing large-particle-size stationary phases. We have found these low-efficiency systems to be unsuitable for our requirement of separating complex mixtures on a relatively small scale. We therefore based our preparative-scale HPLC methods on large bore, conventionally slurry packed columns employing small-particle-size stationary phases. This provided highly efficient columns, well suited to the separation of complex mixtures. Moreover, we were able to make use of existing analytical instrumentation and could directly scale-up analytical separations.

Preparative-scale HPLC separations are usually performed on unmodified sil-
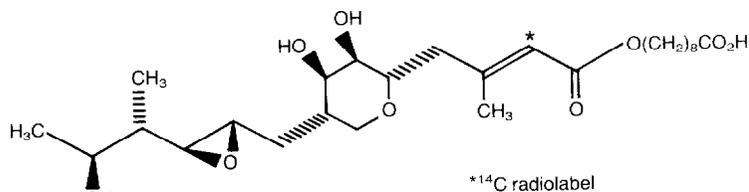
Fig. 1. [2-^{14}C]Mupirocin.

ica using isocratic elution with volatile organic solvents. Such separations have the advantage that they minimise the cost of the stationary phase and pumping system and also allow facile recovery of the purified product. Many pharmaceutical products, however, are not suitable for chromatography using unmodified silica. This is the case for the novel radiolabelled antibiotics [2-^{14}C]mupirocin* (Fig. 1)[4,5], and [thienyl-3-^{14}C]temocillin (Fig. 2)[6]. On a small scale, these acidic antibiotics cannot be satisfactorily purified by conventional purification methods such as thin-layer and open-column chromatography. Moreover, in our experience, even normal phase preparative-scale HPLC has been unsuccessful. These compounds are also sensitive to extremes of pH and therefore only reversed-phase methods of preparative-scale HPLC using buffered aqueous eluents have been successful in their purification. We therefore consider it appropriate to describe our experiences of the purification of these antibiotics to illustrate the use of high-efficiency preparative-scale reversed-phase HPLC techniques employing both isocratic and gradient elution methods.

EXPERIMENTAL

Equipment

Perkin-Elmer Series 3B (flow-rate 0–60 ml min^{-1}, isocratic elution; flow-rate 0–30 ml min^{-1}, gradient elution) or Waters M6000A (flow-rate 0–9.9 ml min^{-1}, isocratic elution) chromatography pumps and Rheodyne 7125 or 7010/11 sample injection valves were used for both preparative- and analytical-scale HPLC. Injector loop volumes of 5 ml and 50 μl were used for preparative and analytical scales respectively. Stainless-steel columns of dimensions 250 mm × 10 mm I.D. (for [2-^{14}C]mupirocin) and 250 mm × 22 mm I.D. (for [thienyl-3-^{14}C]temocillin) were used for the preparative separations. The preparative-scale stainless-steel column fittings employed flow spreading inlets (10 mm I.D. column only) and conical outlets. Analytical-scale
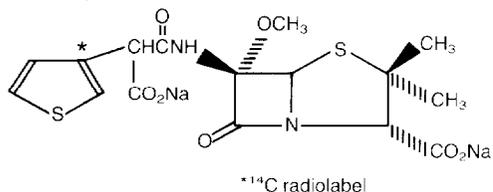
Fig. 2. [Thienyl-3-^{14}C]temocillin.

---

* Previously known as pseudomonic acid A in the published literature. Mupirocin is marketed in the U.K. under the trade mark BACTROBAN.

HPLC was performed using stainless-steel columns of dimensions 300 mm × 3.9 mm I.D. or 250 mm × 4.6 mm I.D. Merck LiChrosorb RP-18 (10-μm mean particle size, part No. 9334), supplied by BDH (Poole, U.K.), was used as stationary phase for some analytical- and all preparative-scale HPLC. Waters μBondapak C_{18} (10-μm mean particle size, part No. 84152) supplied by Waters Assoc. (Northwich, U.K.) was also used as stationary phase for analytical-scale HPLC. All columns, except that of 250 mm × 22 mm I.D. size (kindly supplied by Perkin-Elmer PLC), were packed in our laboratory using a Magnus P6000 HPLC slurry packing unit. A Perkin-Elmer LC75 spectrophotometric detector or Cecil Instruments CE2012 reference-channel variable-wavelength UV monitor were used for UV detection. Narrow bore 1/16 in. O.D. stainless-steel tubing was used for both preparative- and analytical-scale HPLC separations. Radioactivity was measured with a Packard Tri-Carb 2660 liquid scintillation system using Packard Scintillator ES299. Lyophilisation was performed using a Birchover 1-l freeze drier.

Reagents

All reagents used were of either HPLC- or analytical-grade. The water was purified by ion exchange followed by carbon filtration using an Elga B124 water purification unit and Elga Spectrum SC6 cartridge.

Preparative-scale HPLC

[2-14C]Mupirocin. 0.25 *M* Triethylammonium formate buffer solution (pH 4.5)–acetonitrile (70:30, v/v) was used as eluent[7]. Impure [2-14C]mupirocin (*ca.* 50–160 mg) was dissolved in the eluent (5 ml) and loaded by syringe into the injection loop. The sample was injected onto the column using 1.2 loop volumes only, and was chromatographed at a flow-rate of 9.9 ml min⁻¹. A UV detection wavelength of 255 nm was used and the peak corresponding to [2-14C]mupirocin was manually collected as in the example shown in Fig. 3. The eluent containing [2-14C]mupirocin was rotary evaporated *in vacuo* at <30°C to remove the acetonitrile. The remaining aqueous solution was lyophilised overnight. The resultant material was dissolved in ethyl acetate (*ca.* 10 ml) and extracted with saturated sodium chloride solution at pH 3 (*ca.* 10 ml). The saturated sodium chloride solution was extracted with ethyl acetate (3 × 10 ml) and the combined ethyl acetate extracts were dried over anhydrous mag-
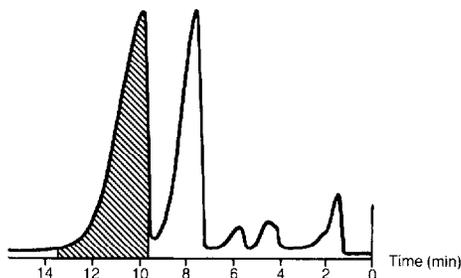

Fig. 3. Preparative-scale HPLC purification of [2-14C]mupirocin. Column: 250 mm × 10 mm I.D., Merck LiChrosorb RP-18 (10 μm). Eluent: 0.25 *M* triethylammonium formate (pH 4.4)–acetonitrile (70:30, v/v). Flow-rate 9.9 ml min⁻¹. Detection: UV at 255 nm. Sample: 58 mg in eluent (5 ml). Shaded area represents fraction collected.

TABLE I

GRADIENT ELUTION OF [THIENYL-3-^{14}C]TEMOCILLIN

Buffer: 0.1 M potassium phosphate buffer solution (pH 6.5).

Time after injection (min)	Eluent composition
0–10	Methanol–buffer (2:98)
10–11	2–15% Methanol, 98–85% buffer (linear)
11–16	15–20% Methanol, 85–80% buffer (linear)
16–26	Methanol–buffer (20:80)

nesium sulphate. The magnesium sulphate was separated by filtration and the filtrate was rotary evaporated *in vacuo* at < 30°C to yield an oil which was dissolved in a minimum volume of ethyl acetate and crystallised following the addition of a small volume of light petroleum (b.p. 40–60°C). The resultant crystalline solid was separated by filtration and residual solvent was evaporated under high vacuum.

[Thienyl-3-^{14}C]temocillin. 0.1 M Phosphate buffer solution (pH 6.5) and methanol were used in a gradient elution system shown in Table I. Impure [thienyl-3-^{14}C]temocillin (*ca.* 90 mg) was dissolved in 0.1 M potassium phosphate buffer solution (4.5 ml) and loaded by syringe into the injection loop. The sample was injected onto the column using 1.2 loop volumes only and was chromatographed at a flow-rate of 25 ml min^{-1}. Using a UV detection wavelength of 254 nm, the peak corresponding to [thienyl-3-^{14}C]temocillin was manually collected as in the example shown in Fig. 4. The eluent containing [thienyl-3-^{14}C]temocillin was rotary evaporated *in vacuo* at < 30°C to remove the methanol and the remaining aqueous solution was acidified to pH 2.0 with 2 M hydrochloric acid in the presence of ethyl acetate (50 ml). The ethyl acetate was separated and the remaining aqueous solution was further extracted with ethyl acetate (2 × 50 ml). The ethyl acetate extracts were combined and dried over anhydrous magnesium sulphate. The magnesium sulphate was removed by filtration and the filtrate was rotary evaporated *in vacuo* to yield an
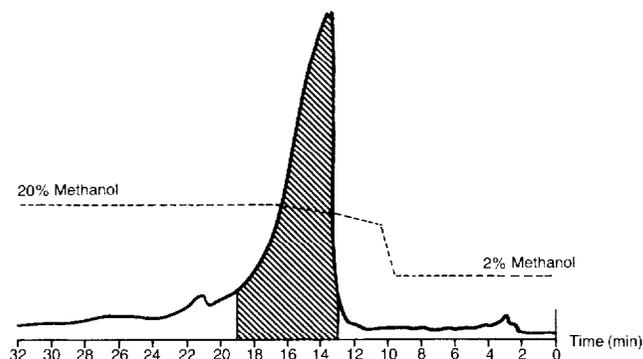

Fig. 4. Preparative-scale HPLC purification of [thienyl-3-^{14}C]temocillin. Column: 250 mm × 22 mm I.D., Merck LiChrosorb RP-18 (10 μm). Eluent: 0.1 M potassium phosphate (pH 6.5)–methanol (2–20% gradient, see Table I). Flow-rate, 25 ml min^{-1}. Detection: UV at 254 nm. Sample: 92 mg in buffer solution (4.5 ml). Shaded area represents fraction collected.

TABLE II

CONDITIONS EMPLOYED FOR ANALYTICAL-SCALE HPLC OF [^{14}C]ANTIBIOTICS

All separations were performed on Waters μBondapak C_{18} stationary phase (column size: 300 mm $\times$ 3.9 mm I.D. or 250 mm $\times$ 4.6 mm I.D.) at a flow-rate of 2 ml min^{-1}.

[^{14}C]Antibiotic	Eluent composition	UV detection (nm)
[2-^{14}C]Mupirocin	0.05 M Ammonium acetate (pH 4.5)–methanol (60:40, v/v)	235
[Thienyl-3-^{14}C]temocillin	0.1 M Potassium phosphate (pH 6.5)–methanol (90:10, v/v)	254

oil which was redissolved in ethyl acetate (7 ml) and water (25 ml) added. The pH of the water was adjusted to 6.0 by the careful addition of 0.1 M and 0.01 M sodium bicarbonate solutions. The aqueous solution was separated and lyophilised overnight to yield [thienyl-3-^{14}C]temocillin.

Analytical-scale HPLC

The radiochemical purities of the ^{14}C-labelled antibiotics were determined by analytical-scale HPLC using the conditions shown in Table II. The chromatographic eluents were fractionated and the amount of radioactivity in each fraction determined by liquid scintillation counting. The fraction of the eluant containing the ^{14}C-labelled antibiotic was determined from the UV absorption. The radiochemical purity was then determined by comparison of the radioactivity collected in the fraction corresponding to the ^{14}C-labelled antibiotic with the total radioactivity from the whole chromatographic run.

RESULTS AND DISCUSSION

Some examples of the preparative-scale HPLC purifications described in this paper are shown in Table III.

[2-^{14}C]Mupirocin was purified using eluent containing 0.25 M triethylammonium formate buffer solution at pH 4.5. This buffer solution was used as it gave a

TABLE III

PREPARATIVE-SCALE HPLC, COMPARISON OF RESULTS

[^{14}C]Antibiotic	Sample loading (mg) on column	Radiochemical purity (%)			Overall recovery (%)
		Original	Purity using prep. HPLC	Purity using coventional methods	
[2-^{14}C]Mupirocin	58	50.2	98.0	97.3	>83
[Thienyl-3-^{14}C]temocillin	92	79.7	94.0	90.1	76

superior chromatographic resolution compared to alternative inorganic buffers and also allowed the possibility of direct removal by lyophilisation[7]. ^{1}H NMR evidence indicated, however, that residual triethylamine remained in the product after lyophilisation. The triethylamine was therefore conveniently removed by acid extraction. This technique has been used to purify many batches of [2-^{14}C]mupirocin at sample loadings between 52 and 110 mg. The example quoted in Table III is the most impure sample that has been purified by a single injection. In this example the method gave an increase in radiochemical purity from 50.2 to 98.0%, with a greater than 83% recovery of [2-^{14}C]mupirocin from the crude mixture. The highest radiochemical purity of [2-^{14}C]mupirocin obtained by this method is 98.8%.

[Thienyl-3-^{14}C]temocillin exists as a pair of diastereoisomers which can, depending upon chromatographic conditions, give rise to a broad single peak or be resolved into a doublet. Several isocratic eluent systems were examined using 250 mm × 10 mm I.D. columns containing either Merck LiChrosorb RP-18 or Waters μBondapak C_{18} stationary phases. Severe overloading effects were observed, however, at column loadings as low as 100 mg. A 250 mm × 22 mm I.D. column containing Merck LiChrosorb RP-18 gave encouraging results using an eluent system containing phosphate buffer solution (pH 6.5)–methanol (98:2, v/v). This system gave a good separation of impurities from [thienyl-3-^{14}C]temocillin which was, however, eluted as a very broad band with a long retention time. The use of gradient elution, increasing the methanol concentration in steps from 2 to 20% (Table I), achieved a satisfactory separation as shown in Fig. 4. [Thienyl-3-^{14}C]temocillin was eluted as a broad doublet containing the pair of diastereoisomers. At a column loading of 92 mg and an original radiochemical purity of 79.7%, [thienyl-3-^{14}C]temocillin was purified to a radiochemical purity of 94.0%, with a 76% recovery from the crude mixture. The ratio of diastereoisomers was unchanged. When purified by preparative-scale HPLC, these antibiotics were obtained with radiochemical purities between 0.7 and 3.9% higher than when purified by slower conventional methods (Table III). We considered this to be a useful and significant increase in radiochemical purity for these types of antibiotics.

At the end of a preparative-scale chromatographic run the system was adequately purged with the organic modifier used in the reversed-phase eluent. In general, after this treatment negligible levels of elutable radioactivity were left on the column, and in the connecting tubing and detector. The sample injection valve usually retained small amounts of radioactivity and was therefore dismantled, washed and reassembled before being used for further separations. After this minimal decontamination procedure the apparatus was suitable for use with other radioactive species.

CONCLUSIONS

The compounds described in this paper are acidic antibiotics which are unstable when exposed to extremes of pH. Their chromatographic characteristics on normal phase silica are poor and attempts at their separation using this type of support were often accompanied by extensive decomposition. Recrystallisation was also usually ineffective. The use of preparative-scale reversed-phase HPLC has allowed the rapid and high yielding purification of these compounds to give products with levels of purity, unobtainable by conventional methods. In particular, their puri-

fication illustrates the utility of high-efficiency preparative-scale HPLC using non-compressed column beds in the purification of radiolabelled pharmaceuticals where the requirement is for the rapid and efficient separation of complex mixtures on a relatively small scale.

REFERENCES

1 D. E. Nettleton, Jr., *J. Liq. Chromatogr.*, 4 (1981) 141.
2 D. E. Nettleton, Jr., *J. Liq. Chromatogr.*, 4 (1981) 359.
3 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, Ch. 15, p. 615.
4 A. T. Fuller, G. Mellows, M. Woolford, G. T. Banks, K. D. Barrow and E. B. Chain, *Nature (London)*, 234 (1971) 416.
5 E. B. Chain and G. Mellows, *J. Chem. Soc. Perkin Trans. 1*, (1977) 294.
6 B. Slocombe, M. J. Basker, P. H. Bentley, J. P. Clayton, M. Cole, K. R. Comber, R. A. Dixon, R. A. Edmondson, D. Jackson, D. J. Merriken and R. Sutherland, *Antimicrob. Agents Chemother.*, 20 (1981) 38.
7 J. Porath, *Nature (London)*, 175 (1955) 478.