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Purification of erucic acid by preparative high-performance liquid chromatography and crystallization

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ABSTRACT

Erucic acid (C22:1 fatty acid) has been found to be useful in the treatment of adrenoleukodystrophy (ALD). It appears to work by reducing the blood levels of very-long-chain fatty acids (VLCFAs) which destroy the myelin sheaths of the nerves. Erucic acid was purified by reversed-phase high-performance liquid chromatography (HPLC) on columns packed with YMC C_{18} (10–20 μm, 120 Å). Using ethanol–water as the mobile phase, the recovery of erucic acid was 69% and the purity was more than 97% as measured by gas chromatography. The amount of saturated VLCFAs was found to be within the limits specified for ALD treatment. The production rate (yield per 8 h shift) was low, however. Using methanol–water instead of ethanol–water as the mobile phase, a ninefold increase in the production rate was achieved. The recovery of erucic acid was 65% and the purity of erucic acid was 98%. All other purity specifications were met. By performing a low-temperature crystallization after the preparative HPLC step, the production rate was increased a further 142%. This represents a 22-fold increase in production rate over the ethanol–water method. The crystalline erucic acid was found to be 99% pure. All other purity requirements were met. The yield for the combined process (HPLC plus crystallization) decreased to 55%, however.

INTRODUCTION

Adrenoleukodystrophy (ALD) is an inherited disease characterized by unusual accumulations of very-long-chain saturated fatty acid (VLCFA) molecules, found in small amounts in the diet and also synthesized in the body. These fats destroy the myelin sheath that insulates and protects the nerves. The symptoms of ALD are memory loss, involuntary motion and death within a few years.

Oral feeding of the monounsaturated fatty acid erucic acid ($\Delta^{13:14}$-docosenoic acid, C22:1) was found to reduce the blood levels of these VLCFAs. A single enzyme was found to be responsible for elongating both long-chain monounsaturated fatty acids and saturated acids whose accumulation is associated with ALD. It was concluded that long-chain monounsaturated fatty acids may work by monopolizing the enzyme, keeping it busy making the apparently harmless unsaturated fatty acids so that the production of the saturated VLCFAs is suppressed [1].

At the request of the United Leukodystrophy Foundation, we undertook to develop a method of purifying erucic acid with the following specifications: (1) the C22:1 content should be $96 \pm 2\%$ in the final product; (2) the C20:1 content should be $\leqslant 2\%$; (3) the saturated C22:0 fatty acid and VLCFA contents should be $< 0.1\%$; and (4) the purified product in the free fatty acid form are preferred to the esters.

In our initial efforts to develop a method which met the above specifications, we used ethanol–water as the mobile phase [2]. Although all purity specifications were met using this solvent system, our efforts in this direction were abandoned when the production rate (defined as the amount of purified erucic acid collected per 8-h shift) proved to be too low.

We found that with methanol–water as the mobile phase, the production rate of erucic acid was greatly increased relative to the method using ethanol–water. This paper reports our efforts to optimize the purification of erucic acid using methanol–water as the mobile phase.

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EXPERIMENTAL

Crude mixtures of fatty acids containing *ca.* 90% [by gas chromatography (GC)] or erucic acid C22:1 (rapeseed oil) was supplied by POS Pilot Plant (Saskatoon, Canada).

For high-performance liquid chromatography (HPLC) for development of the purification methods, the system consisted of a Varian Vista 5500 HPLC, a Rheodyne Model 7125 injector and a Knauer UV detector. The chromatograph used for the larger scale preparative runs was a NovaPrep 5000 from Separations Technology (Wakefield, RI, USA). The data were recorded on a Spectra-Physics integrator and transferred to a personal computer. Fatty acids were detected by UV measurement at 214 nm and by refractive index (RI) detection.

Separations were performed on columns packed at Separations Technology with YMC C_{18} (10–20 μm, 120 Å). For method development a column with dimensions 20 × 0.46 cm I.D. was used. For the initial scale-up, a 20 cm × 1.93 cm I.D. column was used. For the larger scale runs, a 20 cm × 7.5 cm I.D. annular expansion column from Separations Technology was used.

The solvents used were methanol and water (both HPLC grade, Fisher) and were filtered through a 5-μm filter. For the method development and initial scale-up runs performed on the Varian instrument, the solvents were sparged with helium. This was not required for the larger scale runs as the NovaPrep 5000 system uses high-pressure gradient generation. For all injections, 50% (w/v) solutions of the crude fatty acid mixture in methanol–water (95:5) was used.

GC was used to measure the purity of the fractions collected. To facilitate GC analyses, the fatty acids collected were converted into their volatile methyl esters using boron trifluoride–methanol according to the method of Pelick and Mahadevan [3]. The methyl esters were determined by GC on a capillary column. A 50% solution of the methyl ester in hexane (2–4 μl) was used for injection onto the GC column.

Fig. 1 shows the GC trace of a standard solution of fatty acid methyl esters (FAMEs). Note that all the FAMEs are well resolved. Hence GC is a valid method for determining the purity of the collected HPLC fractions.

RESULTS AND DISCUSSION

Two methods were developed to purify erucic acid using methanol–water as the HPLC mobile phase. In the first method reversed-phase HPLC alone was used and in the second reversed-phase HPLC was followed by low-temperature crystallization (LTC).

HPLC-only method

The initial methods development and loading studies were carried out on the 20 cm × 0.46 cm I.D. column. Methanol–water (95:5) was found to be the most suitable composition for this purification. The total run time was 66 min; after 55 min, the column was washed with methanol for 5 min and then re-equilibrated with methanol–water (95:5) for 6 min. After extensive loading studies the maximum load that would give the desired purity was found to be 100 mg. The flow-rate was 0.6 ml/min.

A representative chromatogram is shown in Fig. 2. The erucic acid was collected from 34 to 40 min as shown by the dashed lines. The yield was 65% and the purity by GC was 99%.

The initial scale-up was performed on the 20 cm × 1.93 cm I.D. column. The same mobile phase

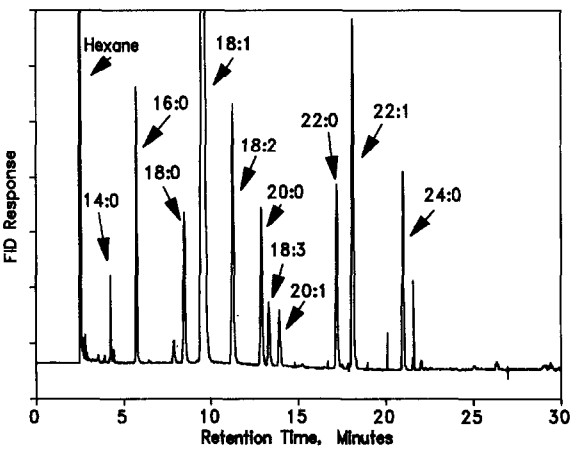

Fig. 1. Gas chromatogram of a standard solution of FAMEs showing that GC resolves the FAMEs of interest and can therefore be used to measure the purity of the collected fractions. Hewlett-Packard Model HP5890 gas chromatograph; column, Supelco SP 2300 fused silica, 30 m × 0.32 mm I.D.; column temperature programmed from 170 to 220°C at 4°C/min; carrier gas, helium. FID = Flame ionization detection.

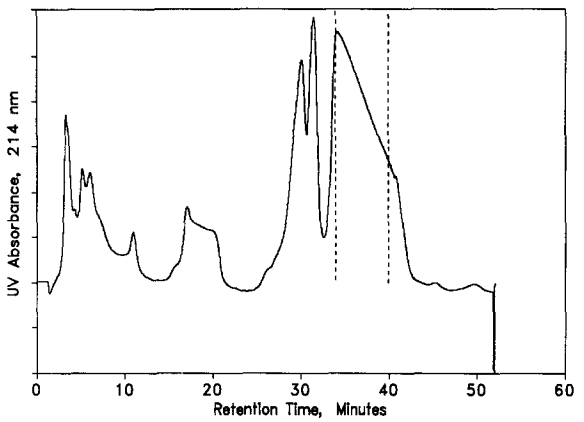

Fig. 2. Method development chromatogram using methanol–water as the mobile phase. Flow-rate, 0.6 ml/min; load, 100 mg; initial mobile phase, methanol–water (95:5); step gradient to 100% methanol at 55 min.

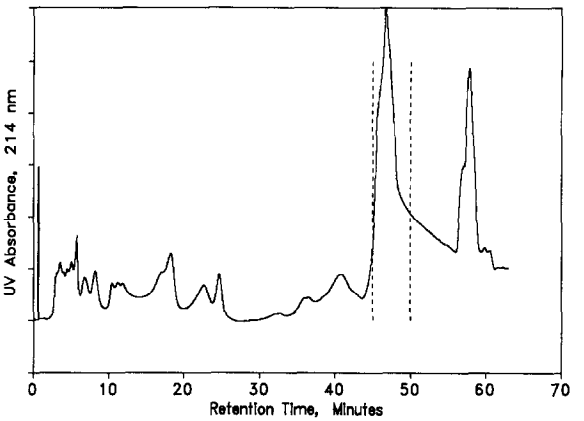

Fig. 3. Initial scale-up on the 20 cm × 1.93 cm I.D. column. Flow-rate, 10.6 ml/min; load, 1.76 g; initial mobile phase, methanol–water (95:5); step gradient to 100% methanol at 55 min.

[methanol–water (95:5)] and methanol were used. In calculating the scale-up parameters for larger columns, the linear velocity is kept constant for each column in order to maintain column efficiency. In such a scale-up, the volumetric flow-rate scales as the square of the column radius, the crude loading scales as the entire volume of the column and the run time and back-pressure scale as the length of the column. The predicted loading, recovery, mobile phase flow-rate and solvent usage for this larger column were calculated using the method development data and are shown in Table I. As seen, the calculated load was 1.76 g and the calculated flow-rate was 10.6 ml/min. To obtain the production rate (grams of erucic collected per 8-h shift) given in Table I, note that each run takes 66 min (1.1 h). Therefore, seven runs can be made in a shift. The production rate is then obtained by multiplying the yield per run in Table I by seven.

Fig. 3 shows a representative chromatogram of the direct scale-up to the 1.93 cm I.D. column using the data in Table I. The erucic acid is in the large peak that elutes near 48 min. A comparison of Figs. 2 and 3 will show that the retention time for the erucic acid peak is longer on the 1.93 cm I.D. column than on the 0.46 cm I.D. column, *i.e.*, the 1.93 cm I.D. column is more efficient. We were able, therefore, to increase the load above that calculated in Table I from the method development data. We therefore increased the load from 1.76 to 2 g. As this amount of overloading causes a decrease in retention time, it was possible to decrease the flow-rate (thereby further increasing the efficiency) without decreasing the production rate. Hence the flow-rate was reduced from 10.6 to 6.0 ml/min, and the gradient conditions were changed to those in Table II. A representative chromatogram is shown in Fig. 4.

In an early experiment, fifteen fractions were collected. Each fraction was dried by rotary evaporation and converted to the methyl ester for GC analysis. The GC analysis showed that fractions 9, 10, 11 and 12 collected between retention times of 25

TABLE I

CALCULATED SCALE-UP PARAMETERS FOR THE 20 cm × 1.93 cm I.D. COLUMN

Column length × I.D. (cm)	Load (g per run)	Yield (g per run)	Yield (%)	Production rate yield (g per shift)	Flow-rate (ml/min)
20 × 0.46	0.100	0.065	65%	0.46	0.6
20 × 1.93	1.76	1.14	65%	7.98	10.6

TABLE II

GRADIENT CONDITIONS FOR FIGS. 4 AND 6

Mobile phase flow-rate, 6.0 ml/min.

Time (min)	A[a] (%)	B[a] (%)
0	100	0
50	100	0
51	0	100
60	0	100
61	100	0
66	100	0

[a] A = methanol–water (95:5, v/v); B = methanol.

and 36 min each contained 97–99% of erucic acid and met the other purity specifications of the project. Fraction 13, collected between 36 and 40 min, contained 92.2% of erucic acid, but it also contained more than 1% of saturated C24:0 fatty acid. All the fractions collected after 36 minutes contained an increasing amount of long chain saturated fatty acid, especially C24:0 saturated fatty acid. We therefore performed a run in which one fraction was collected between 25 and 36 min, shown by the dashed lines in Fig. 4. This fraction gave a yield of 65% and 1.3 g of erucic acid were recovered. GC analysis of the methyl ester showed it to be 98% pure erucic acid. All other purity specifications were also met.

Note that two types of traces are shown in Fig. 4, obtained by RI and UV detection. The size of an RI peak is about proportional to the concentration of the compounds in the peak. Thus, in Fig. 4, the RI trace shows that most of the erucic acid lies between retention times of 25 and 40 min. The intensities of the UV peak are determined by electronic transitions within the molecules responsible for the peaks. The relative intensities of two peaks, therefore, are not necessarily related to the relative concentrations of the compounds in the peaks. Thus the UV trace in Fig. 5 shows that there is a compound with a retention time near 20 min, and the RI trace shows that this compound is in low concentration.

In an effort to increase the production rate, we switched to methanol as mobile phase at 36 min, as the collection of erucic acid is completed at this point. This method was chosen as the optimum HPLC-only method. The chromatographic conditions are given in Table III and the chromatogram is shown in Fig. 5. Unfortunately, the peak near 50 min retention time tailed to such an extent that the length of the run was not decreased significantly. Thus switching to methanol at 36 min decreased the total run time by only 1 min.

Further work showed that when the load was increased to more than 2.0 g for this column, the purified erucic acid contained long-chain fatty acids, especially the C24:0 which elutes after erucic acid, due to the tag-along effect [4–6]. In order to main-

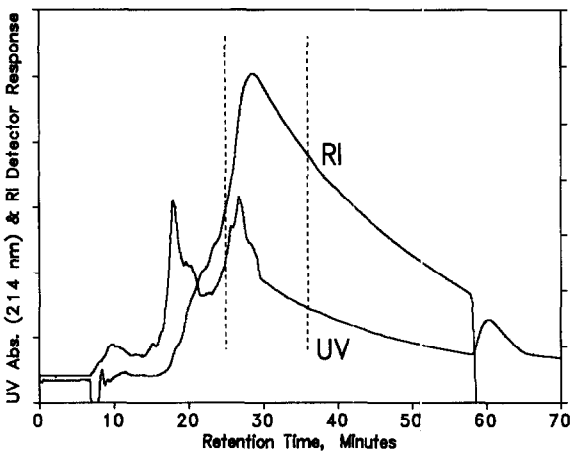

Fig. 4. Increased load on the 20 cm × 1.93 cm I.D. column. Flow-rate, 6.0 ml/min; load, 2.0 g; step gradient to 100% methanol at 50 min. Other conditions as in Table II.

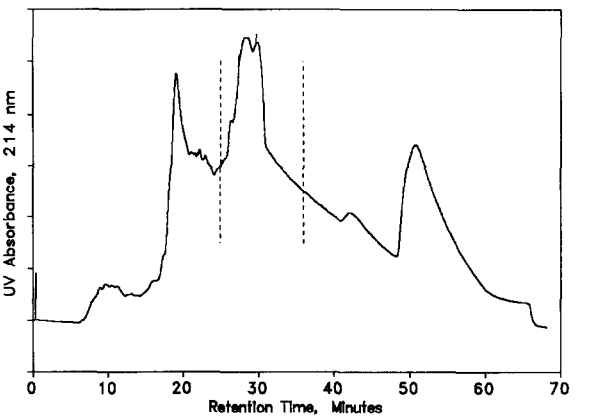

Fig. 5. Optimized chromatogram of HPLC-only method on the 20 cm × 1.93 cm I.D. column. Flow-rate, 6.0 ml/min; load, 2.0 g; step gradient to 100% methanol at 35 min. Other conditions as in Table III.

TABLE III

GRADIENT CONDITIONS FOR THE OPTIMIZED HPLC-ONLY METHOD (FIG. 5)

Column dimensions, 20 cm × 1.93 cm I.D.; load, 2 g; mobile phase flow-rate, 6.0 ml/min.

Time (min)	A[a] (%)	B[a] (%)
0	100	0
35	100	0
36	0	100
55	0	100
56	100	0
65	100	0

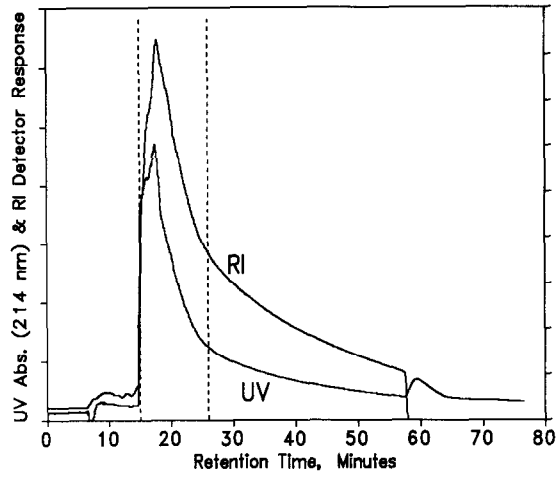

Fig. 6. Initial chromatogram of HPLC–LTC method on the 20 cm × 1.93 cm I.D. column. Flow-rate, 6.0 ml/min; load, 4.0 g; step gradient to 100% methanol at 50 min. Other conditions as in Table II.

tain the desired purity, a narrower fraction would have to be collected. Thus the production rate would be lowered.

HPLC followed by low-temperature crystallization (HPLC–LTC method)

Low-temperature crystallization was investigated as a purification technique for the fatty acids. Several crystallizations were performed on the crude products and on the fractions obtained from the HPLC runs. The crystals and the mother liquors were converted into the methyl esters and analyzed by GC for their fatty acid composition.

At the temperatures used in the crystallization (5–7°C), the fatty acids with chain lengths shorter than erucic acid remained in solution and were thus easily separated by filtering. However, erucic acid and the VLCFAs, because of their higher melting points, crystallized together.

The crystallization results prompted us to attempt the purification by combining HPLC and LTC. We hypothesized that the loading could be substantially increased if the HPLC run were followed by the crystallization step. The HPLC run should remove the later eluting VLCFAs, and the crystallization should remove the earlier eluting, shorter chain fatty acids.

To test this hypothesis, the load was increased to 4 g. The gradient conditions were the same as used in Fig. 4 and are given in Table II. Fig. 6 shows the HPLC run. A comparison with Fig. 4 shows a reduction in retention time and a loss of resolution. After several experiments it was determined that the fraction between 15 and 26 min, shown by the dashed lines in Fig. 6, would yield acceptable purity results. This fraction was collected and kept at 5–7°C in an ice-bath. The crystalline product obtained was filtered under suction in a Buchner funnel using a glass-fiber filter. The crystals were analyzed by GC for their fatty acid composition and purity. The purity of the erucic acid was found to be greater than 99%, and the method met all the other purity specifications. However, the overall yield of erucic acid from the HPLC–LTC procedure was only 55%.

For the final HPLC–LTC method, we switched to methanol as mobile phase at 26 min, since the collection was completed at this point. This decreased the run time significantly from 66 to 46 min, thus increasing the production rate. The chromatographic conditions for the optimized HPLC–LTC method are given in Table IV and the chromatogram is shown in Fig. 7. Again the erucic acid peak was collected from 15 to 26 min, as shown by the dashed lines.

Summary of the 20 × 1.93 cm column results

Table V gives a summary of the results obtained on the 20 cm × 1.93 cm I.D. column. The run times include the time necessary to equilibrate the column between runs. Note that as the run time for the

TABLE IV

GRADIENT CONDITIONS FOR THE OPTIMIZED HPLC–LTC METHOD (FIG. 7)

Column dimensions, 20 × 1.93 cm I.D.; load, 4 g; flow-rate, 6.0 ml/min.

Time (min)	A[a] (%)	B[a] (%)
0	100	0
26	100	0
27	0	100
40	0	100
41	100	0
46	100	0

[a] A = methanol–water (95:5, v/v); B = methanol.

HPLC-only method is slightly longer than 1 h, seven HPLC-only runs could be made in an 8-h shift. Thus 9.1 g of erucic acid could be produced in an 8-h shift (1.3 g × 7 runs). This represents a ninefold increase in the production rate (grams collected per shift) compared with the method with ethanol–water as the mobile phase.

The run time for the HPLC–LTC method is 0.77 h; hence ten of these runs could be made in an 8-h shift. This leads to a production rate of 22 g of erucic acid collected per 8-h shift (2.2 g × 10 runs). This represents a 22-fold increase in the production rate relative to the ethanol–water method. The time needed for the crystallization step was less than 30 min. Hence at this scale, the production rate is controlled by the HPLC step. That is, since the crystallization step can be carried out simultaneously with the next HPLC run, and since the HPLC run takes a longer time (0.77 h, Table V), the average time for the entire HPLC–LTC procedure will be 0.77 h once a steady production stream is established.

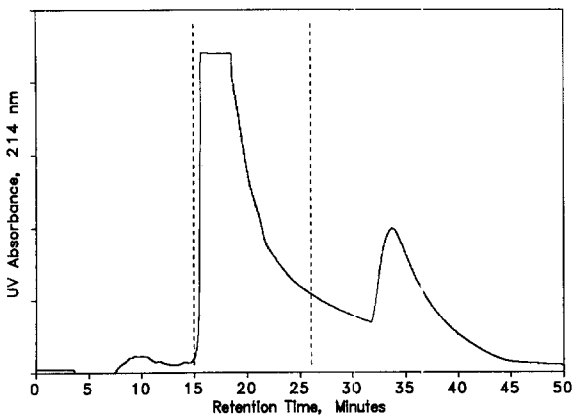

Fig. 7. Optimized chromatogram of HPLC–LTC method on the 20 cm × 1.93 cm I.D. column. Flow-rate, 6.0 ml/min; load, 4.0 g; step gradient to 100% methanol at 26 min.

TABLE V

SUMMARY OF OPTIMIZED HPLC METHODS ON THE 20 cm × 1.93 cm I.D. COLUMN USING METHANOL–WATER

Parameter	Method	
	HPLC-only	HPLC/LTC
Run time (h)	1.08	0.77
Methanol usage (l per run)	0.377	0.265
Methanol usage (l per shift)	2.64	2.65
Load (g per run)	2.0	4.0
Load (g per shift)	14	40
Yield (g per run)	1.3	2.2
Yield (g per shift)	9.1	22
Yield (%)	65	55
Erucic acid purity (%)	98	>99
C24:1 content (%)	0.01	0.02
C20:1 content (%)	0.04	0.03
C16:0–C20:0 content (%)	0.04	0.06
C22:0 and longer saturated VLCFA content (%)	0.045	0.041

Note also that even though more runs are made per shift with the HPLC–LTC method, the methanol consumption per shift is about the same as for the HPLC-only method. Thus, in determining whether the method is acceptable, one should consider production rate, yield and methanol consumption per gram of purified erucic acid.

Scale-up to 20 cm × 7.5 mm I.D. annular expansion column

We scaled up the HPLC–LTC method, which we consider to be the better of the two methods, to the 20 cm × 7.5 cm I.D. annular expansion column. The calculated scale-up parameters are given in Table VI and the chromatogram is shown in Fig. 8. The gradient conditions were the same as those given in Table V. The erucic acid fraction was collected from 7 to 26 min as shown by the dashed lines in Fig. 8. After low-temperature crystallization, the purity of this fraction was found by GC to be

TABLE VI

CALCULATED SCALE-UP PARAMETERS FOR THE HPLC–LTC METHOD ON THE 20 cm × 7.5 cm I.D. ANNULAR EXPANSION COLUMN

Parameter	Value	Parameter	Value
Run time (h)	0.77	Yield (g per shift)	332
Load (g per run)	60.6	Flow-rate (ml/min)	90.6
Load (g per shift)	604	Methanol usage (l per shift)	32.9

98.8% and the yield was 59%. The levels of the other fatty acids were below those specified in the Introduction.

In comparison with the HPLC–LTC run on the 20 cm × 1.93 cm I.D. column (Fig. 7), both peaks have shifted to shorter retention times on the 20 cm × 7.5 cm I.D. column (Fig. 8). This indicates that the larger column is more overloaded than the 20 cm × 1.93 cm I.D. column. Hence the run time is slightly shorter than predicted in Table VI. With this reduction in run time, it appears that one or two more runs could be made in an 8-h shift, thus further increasing the production rate.

CONCLUSIONS

We have shown that preparative HPLC can be used to purify erucic acid from rapeseed oil to a level that could be used in clinical trials to treat adrenoleukodystrophy. Using ethanol–water as the mobile phase, the production rate was too low to be of practical significance. With methanol–water as the mobile phase, the production rate is much higher. When low-temperature crystallization is combined with HPLC, the production rate is more than doubled in comparison with HPLC alone. It appears that the HPLC–LTC method, on scale-up to larger systems, could be used to produce enough crucic acid for clinical trials. However, further engineering is needed (in such areas as solvent recycling) to make the method economically feasible.

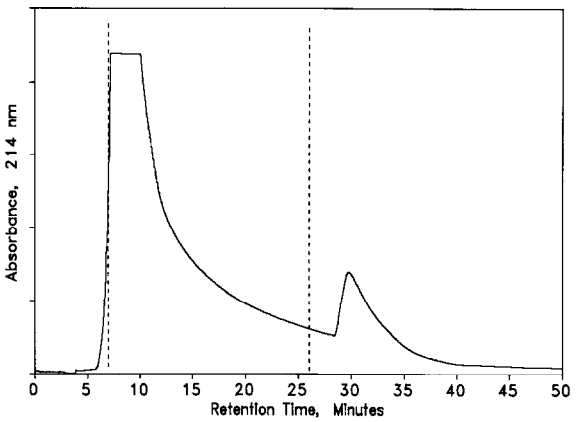

Fig. 8. Chromatogram of HPLC–LTC method scaled-up to the 20 cm × 7.5 cm I.D. annular expansion column. Flow-rate, 90.6 ml/min; load, 60.4 g.

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