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Separation and detection of oxidation products in Neurolite raw material

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ABSTRACT

N,N'-1,2-Ethylenediylbis-L-cysteine diethyl ester (ECD) is a chiral compound and is a key component of Neurolite, a brain imaging agent. In order for the material to be used in the manufacture of Neurolite, it must meet optical rotation and other purity specifications. The optical rotation of ECD is affected by the presence of oxidation-related impurities of the parent material. Prior to this work, the optical rotation was used as a gross indication of these impurities. During product development, information regarding the impurity profile became necessary in order to understand and monitor ECD degradation. A gradient elution high-performance liquid chromatographic method compatible with liquid chromatography–mass spectrometry was developed and optimized using Drylab G software. System suitability of the method was assessed by adding L-methionine ethyl ester and acetophenone to the ECD standard as resolution markers. Comparison of the resolution between each marker and ECD with previous measures of resolution ensures sufficient zone capacity to resolve all potential impurities.

INTRODUCTION

Pharmaceutical quality and regulatory considerations have spurred the need to separate and identify potential impurities and degradation products in pharmaceuticals. As a result, high-performance liquid chromatography (HPLC) is becoming increasingly popular as in many instances the active ingredient and the impurities can be separated and determined using the same method. The past two decades have seen advances in both the theory and practice of HPLC which have allowed greater control and reproducibility of methods.

Concurrently, optimization theory has been developed and shown to be applicable to the development of all types of analytical methods. The optimization of HPLC methods has thus moved from a tedious and time-consuming exercise to one amenable to computer control and simulation. The role of computers in chromatographic method development has been reviewed [1–3]. One computer system for HPLC method development is DryLab, which has been described in detail [4]. DryLab is computer software which helps optimize an HPLC method using an approach that an experienced chromatographer would employ. It is based on the observation that changes in mobile phase concentration offer the greatest potential for separation improvements in HPLC. The program requires that the user supply retention times and peak identities for a given sample from three experimental runs in which only one

mobile phase variable is changed; the program will then pick the optimum mobile phase composition. Optimization of other separation variables, such as other mobile phase components and different columns, may be achieved with only a few additional experimental runs.

DryLab has been successfully applied to various real-life analytical problems [5–7]. DryLab G [8] is an extension of the DryLab software which permits the optimization of gradient HPLC parameters: gradient steepness and the initial and final percentages of each solvent. DryLab G was used to develop a method for detection of oxidation impurities in N,N′-1,2-ethylenediylbis-L-cysteine diethyl ester (ECD), a chiral compound.

An important aspect of method optimization is the definition of the goal of the method. For our purposes, the following were desired: adequate resolution between the major component (ECD) and observed degradation products; resolution (>1.5) of all known impurities; minimized run time without sacrificing resolution; and rugged performance.

Gradient elution HPLC was chosen in order to resolve adequately all peaks of interest and to provide reasonable peak capacity. A large zone capacity will give the best chance of resolving all potential impurities. Although gradient elution was chosen as the initial method, a secondary goal was to develop an isocratic method if possible. The experimental design that was used permitted an extension to isocratic separation. The mobile phase components were picked for compatibility with liquid chromatography–mass spectrometry (LC–MS) to aid impurity identification. Minimum run time, although desired, was not critical to this method as it was designed for evaluation of only a few batches of raw material each year. Thus, analysis time could be sacrificed for greater resolving power. Finally, in order to ensure proper functioning of the method in various quality control laboratories, a system suitability assessment was developed.

EXPERIMENTAL

Instrumental

The reversed-phase gradient method was performed using two different HPLC instruments with UV detection at 210 nm: a Hewlett-Packard (Avondale, PA, USA) Model 1090L with a filter photometric detector and a Spectra-Physics (San Jose, CA, USA) Model SP8785 LC with a Model 1000S diode-array detector (Bioanalytical Systems, West Lafayette, IN, USA). Both instruments were connected to Hewlett-Packard Model 3392 integrators. The Spectra-Physics instrument was also connected to a Model CR/DS Flow-One beta radioactive flow detector (Radiomatic Instruments, Tampa, FL, USA). Separations were carried out on 25 cm × 0.46 cm I.D. Zorbax Rx columns and 1.25 cm × 0.4 cm Zorbax Rx guard cartridges (Mac Mod Analytical, Chadds Ford, PA, USA) with a flow-rate of 1.5 ml/min at room temperature. The total run time was 30 min, allowing for adequate equilibration of the column.

Chemicals

The mobile phase was a mixture of aqueous ammonium chloride (Fisher Scientific, Fairlawn, NJ, USA) and acetonitrile (Mallinckrodt, Paris, KY, USA). Deionized water was prepared with a Milli-Q water purification system (Millipore, Milford,

MA, USA). For LC–MS compatibility ammonium chloride was the buffer of choice. All solvents used for the gradients contained 0.1 *M* ammonium chloride, so that only the organic composition was varied over the gradient time. The dwell time for the system was determined to be 1.0 min according to procedures described [2].

ECD · 2 HCl was prepared according to published procedures [9,10]. [^{14}C]ECD was purchased from DuPont NEN (Boston, MA, USA). L-Methionine ethyl ester hydrochloride and acetophenone (Aldrich, Milwaukee, WI, USA) were used as received.

Computer software

The DryLab G software (LC Resources, Lafayette, CA, USA) was used on an IBM PS/2 Model 50 Z personal computer.

Experimental design

To facilitate the use of DryLab in optimization, and to aid in the identification of peaks, a 4 mg/ml solution of [^{14}C]ECD was used. The solution of [^{14}C]ECD was placed at room temperature for 24 h to form a large amount of oxidation products. Following injection of 25 μl of this [^{14}C]ECD solution a gradient was run from 20 to 90% acetonitrile for 20 min to determine the mobile phase strength required to elute all peaks of interest. The resulting chromatogram (Fig. 1) shows that no other peaks elute at acetonitrile concentrations above 50%. This indicates that there is no need to go above 50% acetonitrile in future optimization steps. The [^{14}C]ECD also contains impurities that are not found in the actual ECD raw material. These impurities are a result of the [^{14}C]ECD synthesis process, which is very different from that of the actual raw material, and were discounted in the optimization, as noted below. As it is easy to detect low levels of [^{14}C]ECD degradation products that might not have good UV absorbance, [^{14}C]ECD was used to ensure that all oxidation products were visible.

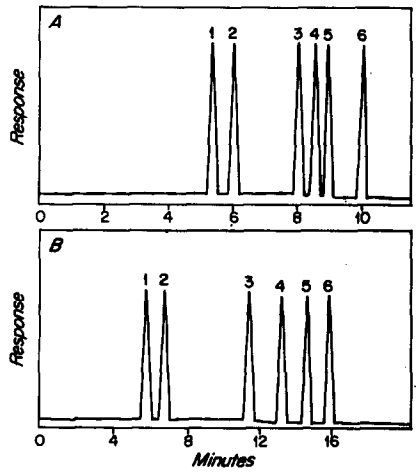

Fig. 1. Stimulated elutions using DryLab G. Chromatograms simulated using data from Table I. (A) 20–90% acetonitrile gradient in 20 min; (B) 20–50% acetonitrile gradient in 20 min.

RESULTS AND DISCUSSION

Optimization

In order to predict gradient elution times accurately data are required from at least two experimental runs which differ in gradient time. In this instance, a 20–50% acetonitrile gradient was run with gradient times of 10 of 60 min. The results from these runs are shown in Fig. 2 and Table I. The experimental conditions and retention data were entered into the computer to perform optimization on the gradient. Data from the experimental runs indicated that there are four peaks of interest, *i.e.*, ECD

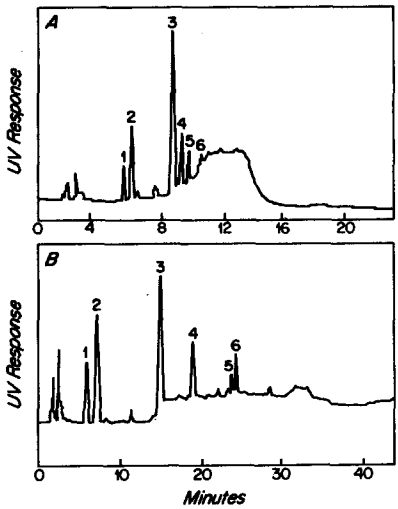

Fig. 2. Separation of oxidized [^{14}C]ECD material using gradient elution. Column, 25 × 0.46 I.D. cm Zorbax Rx; mobile phase gradient from 20 to 50% acetonitrile–aqueous ammonium chloride, with flow-rate 1.5 ml/min, temperature = 25°C and [^{14}C]ECD data from Table I. HPLC with gradient times of (A) 10 and (B) 60 min.

TABLE I

RETENTION TIMES OF PEAKS OF INTEREST

Peak No.	Retention time (min)	
	10-min run	60-min run
1	5.65	6.06
2	6.33	7.17
3[a]	8.59	14.78
4	9.22	18.75
5	9.69	21.84
6	10.79	23.10
Critical pair[b]	4 and 5 (R_s = 2.45)	5 and 6 (R_s = 2.02)

[a] ECD peak.

[b] The critical pair are the two closest eluting peaks for any given conditions.

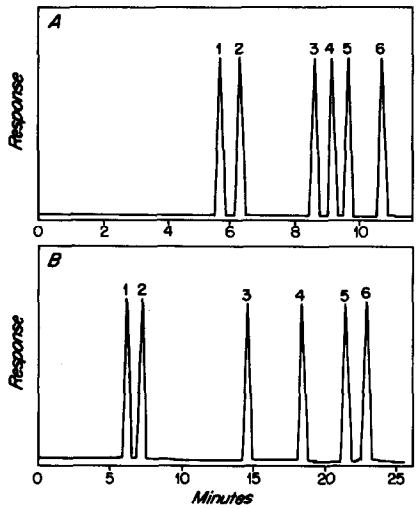

Fig. 3. Simulated elutions of oxidized [^{14}C]ECD material using gradient elution. Simulations using DryLab G from the data in Table I with gradient times of (A) 10 and (B) 60 min.

(peak 3) and three oxidation peaks (4, 5 and 6). In addition, two impurities were observed from the [^{14}C]ECD which were not found in the ECD raw material itself. The retention times of these six peaks were entered into DryLab as shown in Table I.

The computer program simulates a hypothetical picture for each change in run time or gradient step (Fig. 3). This information is used to predict the best conditions for adequate resolution of the critical pair. The first step is to change the run time using the same gradient. The program will calculate the average resolution, critical pairs and the retention time of the last-eluting peak. Snyder *et al.* [8] indicated that the accuracy between the actual and predicted retention time is ± 1–3% and 5–10% for resolution. Table II lists the calculated values as a function of changing the gradient time for 20–50% gradient.

The data show that the average resolution for each variation in gradient time is very good. Therefore, we need to examine each of the critical pairs more closely. Good separation of the ECD peak (3) and the three oxidation peaks (4, 5 and 6) is most important to this method. The first two peaks are of no consequence as they appear only in the batch of [^{14}C]ECD and not in actual ECD raw material. The gradient times between 7.3 and 16.6 min and from 30.3 to 62.6 min have critical pairs that include the three oxidation peaks (4, 5 and 6). Therefore, these gradient times are excluded from consideration. Between 17.7 and 24.6 min, the critical pair is 1 and 2, the two [^{14}C]ECD impurities. Therefore, the best gradient times on which to focus attention are between 17 and 25 min. A gradient time of 17 min was chosen as a compromise between best resolution of the known impurities, reasonable run time and excess resolving power to facilitate detection of other potential impurities.

The next step is to determine if any time can be saved at the beginning or end of the gradient by changing the gradient step. DryLab information led to a final choice of a 20–44.5% acetonitrile gradient. Fig. 4 shows the DryLab and the actual chromatogram using the optimized conditions. The predicted and actual retention times are

TABLE II

DRYLAB PREDICTIONS FOR A 20–50% ACETONITRILE GRADIENT WITH VARYING GRADIENT TIME

Gradient time (min)	Minimum R_s[a]	Critical band pair	Retention: last t_R (min)	k' (av.)[b]
7.3	1.89	4, 5	9.25	2.93
8.3	2.12	4, 5	9.86	3.20
9.5	2.35	4, 5	10.51	3.50
11.5	2.71	4, 5	11.56	4.00
12.3	2.83	4, 5	11.94	4.18
14.6	3.14	4, 5	12.92	4.66
15.6	3.26	4, 5	13.33	4.86
16.6	3.38	4, 5	13.74	5.08
17.7	3.44	1, 2	14.17	5.30
18.9	3.48	1, 2	14.60	5.52
20.2	3.52	1, 2	15.04	5.76
24.6	3.63	1, 2	16.42	6.51
30.3	3.49	5, 6	17.93	7.39
34.6	3.20	5, 6	18.91	7.99
40.5	2.86	5, 6	20.10	8.74
46.2	2.57	5, 6	21.10	9.40
52.6	2.29	5, 6	22.11	10.09
54.0	2.23	5, 6	21.80	9.88
61.6	1.96	5, 6	23.30	10.95

[a] R_s = resolution.

[b] k' (av.) = Average capacity factor.

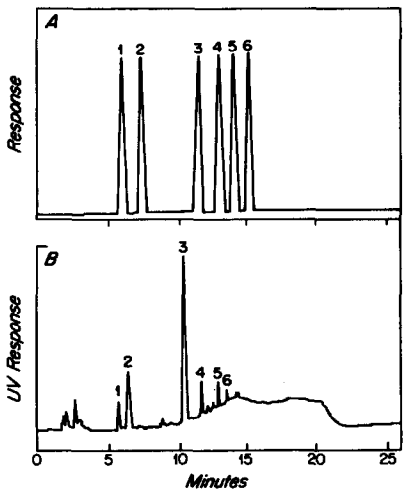

Fig. 4. Comparison of DryLab G prediction *vs.* actual gradient: Conditions as in Fig. 2, except gradient is from 20 to 44.5% acetonitrile in 17 min. (A) Simulated chromatogram; (B) actual chromatogram.

TABLE III

ACTUAL RETENTION TIMES *VS.* DRYLAB-PREDICTED RETENTION TIMES

Peak No.	Actual retention time (min)	Predicted retention time (min)	Difference (%)
1	5.65	5.89	−4.2
2	6.33	6.80	−7.9
3	10.23	11.16	−9.1
4	11.55	12.73	−10.2
5	12.76	13.86	−8.6
6	14.20	15.24	−7.3

given in Table III. The peak elution order was assumed to be constant throughout; the reasonable agreement between predicted and actual retention times indicates that this is a good assumption and that more stringent peak tracking methods are not required. The actual chromatogram in Fig. 4 contains the extra [^{14}C]ECD peaks and a baseline shift. By running a water blank with the gradient, it was determined that the shift in baseline and a very small blip at the void volume are due to the gradient.

System suitability

System suitability is used to ensure reproducibility and proper functioning of a method each time it is run. As resolution is the most important criterion of the method, two compounds are added to the ECD standard and are used as resolution markers. The compounds, L-methionine ethyl ester and acetophenone, have retention times that bracket the working range of the gradient. A straightforward calculation of resolution between each of these compounds and ECD ensures that the method is working properly.

To measure system suitability, five injections of an ECD standard containing the markers are used. Table IV gives the measured retention times of each component in the standard, and the measured resolution with respect to ECD.

Chromatography of impurities using the optimized method

This purity-indicating gradient method was developed to detect and resolve oxidation products that affect the optical rotation of the ECD · 2HCl raw material.

TABLE IV

RETENTION TIMES AND RESOLUTION OF MARKERS

n = 4; analyzed in 6 days.

Component	Retention time range (min)	Resolution (compared with ECD)
L-Methionine ethyl ester	2.6 ± 0.02	8.5 ± 0.77
ECD	6.7 ± 0.03	–
Acetophenone	12.9 ± 0.05	10.4 ± 1.55

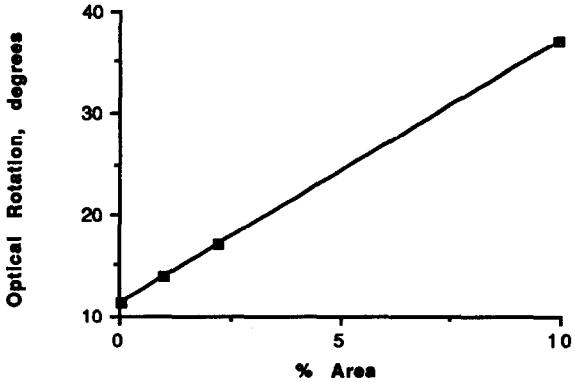

Fig. 5. Correlation of optical rotation and purity analysis of ECD. % Area = total area of impurities from Table V. The linear regression of the data shows a correlation of (optical rotation) = 11.2 + 2.6912x, with correlation coefficient = 1.000.

The raw material can be synthesized in high purity as the L,L-isomer, which has an optical rotation of +11°, and a chiral HPLC method has been developed to determine optical purity of the raw material [11]. Oxidation of the raw material will cause the optical rotation to increase. Three main oxidation impurities are typically seen, and the summed area of impurity peaks can be related to the percentage optical rotation as shown in Fig. 5 and Table V. The amount of oxidation impurities also increases with increasing age of the ECD solution. Efforts to isolate these oxidation products have been attempted without success owing to the complicated chemistry that can occur with dithiol–diamine materials. The oxidation products represent a very small amount of the original ECD · 2HCl, hence this technique is very sensitive to small changes in the quality of ECD · 2HCl.

We have also found excellent resolution for impurities other than those produced by oxidation. Fig. 6 shows the chromatography of a lactam formed by cyclization, a cleavage product (AECEE) and a thiazolidine impurity (EMT).

TABLE V

DETECTION OF OXIDATION PRODUCTS BASED ON OPTICAL ROTATION

Purity	Optical rotation (°)	Area of oxidation impurities (%)
High	11.3	0.03
Medium	13.9	1.01
Low	17.0	2.23
Low	37.0	9.94

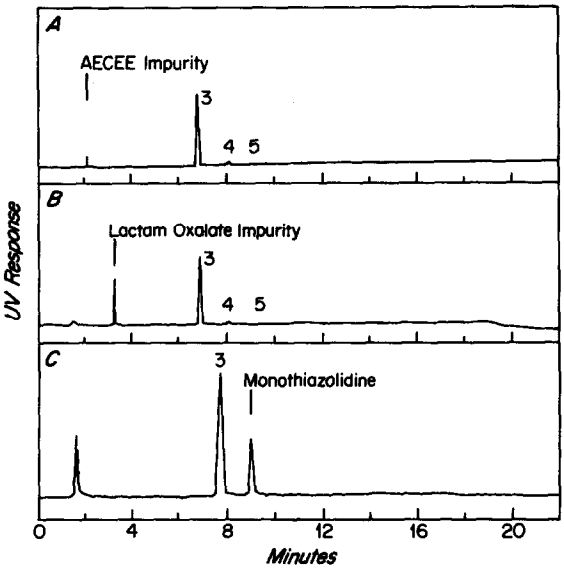

Fig. 6. Detection of impurities other than those produced by oxidation: (A) N-(2-aminoethyl)cysteine ethyl ester (AECEE), a cleavage product; (B) lactam oxalate formed by cyclization; (C) monothiazolidine (EMT). Peaks 3 is ECD and peaks 4 and 5 are two oxidation products.

CONCLUSION

In order for ECD to be used in the manufacture of Neurolite brain imaging agent, the material must meet optical rotation and purity specifications. As the optical rotation is affected by oxidation products, a method for the specific determination of the impurities was developed. A reversed-phase gradient was used to maximize the ability to find and eventually identify all oxidation products and also any potential impurities that may arise in the future. We were able to meet all our goals and reduce the development time with the use of DryLab. The resulting HPLC method is more specific than previous methods and provides excellent resolution of all potential impurities.

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