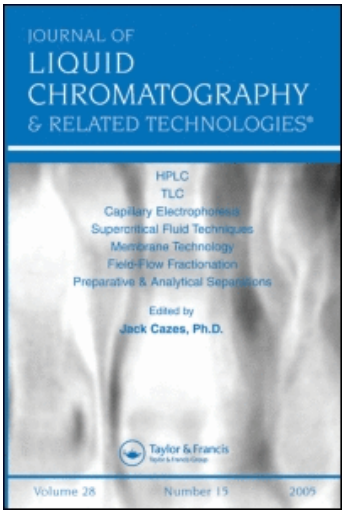

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-speed LC Analysis Using Electrochemical Detection

J. M. Di Bussolo[a]; M. W. Dong[a]; J. R. Gant[a]
[a] The Perkin-Elmer Corporation Norwalk, Connecticut

To cite this Article Bussolo, J. M. Di , Dong, M. W. and Gant, J. R.(1983) 'High-speed LC Analysis Using Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 6: 12, 2353 — 2373
To link to this Article: DOI: 10.1080/01483918308064911
URL: http://dx.doi.org/10.1080/01483918308064911

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(12), 2353-2373 (1983)

HIGH-SPEED LC ANALYSIS USING ELECTROCHEMICAL DETECTION

J.M. Di Bussolo, M.W. Dong and J.R. Gant

The Perkin-Elmer Corporation
Norwalk, Connecticut 06855

ABSTRACT

The chromatographic performance of an electrochemical detector incorporating a flowcell with improved dispersive characteristics has been evaluated for use in high-speed liquid chromatography. High-speed C18/3 μm columns, 100 x 4.6 mm, i.d. were found to be well matched to this detector with respect to extra-column contributions to band broadening. The capabilities of this high-speed LC-EC system are demonstrated by a 3-minute separation of phenols and a 4-minute separation of catecholamines and acetominophen.

INTRODUCTION

In 1981, several publications by DiCesare et al. provided an introduction to practical high-speed liquid chromatography (LC) (1,2). The importance of considering all components of the LC system, in order to optimize system performance, was clearly demonstrated in studies carried out examining the relationship between column geometry and the extra-column contributions to

 0148-3919/83/0612-2353$3.50/0

peak dispersion, the so-called instrumental bandwidth (IBW). As a result of this effort, the first commercial LC system designed for high-speed analysis became available (1).

Important features of this system include a low dispersion injector, low volume (2.4 µL originally, now 1.4 µL) detector flowcell, fast detector response time (135 milliseconds originally, now 20 milliseconds) and short, 0.007 inch i.d. connecting tubes. This system is compatible with short columns (e.g. 100 or 33 mm long by 4.6 mm i.d.) packed with 3 µm particles. Reduction in particle size from 10 µm or 5 µm to 3 µm provides two main advantages. As particle size decreases, the column length required to achieve a given efficiency decreases proportionally. Also as particle size decreases, the optimum mobile phase velocity increases. Therefore, shorter columns at higher flow rates achieve similar separations in reduced times as discussed below.

The intervening years have seen rapid growth (3-10) in the acceptance of high-speed LC and significant improvements over initial systems in several respects. Specifically, further reductions in instrumental bandwidth contributions of connecting tubing (11), detectors (12), and injectors (13) are responsible for these advances.

All of these early developments occurred using the most commonly encountered variable wavelength UV detector. A similar path of development is being traversed by fluorometric (14) and RI detection (15) where IBW values of 40 and 55 µL, respectively, have been reported. To-date, little has been reported with respect

to high-speed liquid chromatography with electrochemical (EC) detection (16). However, many practical applications (e.g. 17-20) and several good discussions of basic detection principles (21, 22) have been published using EC detection. The purpose of this communication is to report the results of preliminary investigations of the high-speed capabilities of LC-EC.

EXPERIMENTAL

All chromatographic equipment used in this study is available from The Perkin-Elmer Corporation (Norwalk, CT). Peak dispersion studies and high-speed LC of catecholamines were performed with a SERIES 10 pump. Other high-speed analyses were performed with the SERIES 4 Liquid Chromatograph. Injections were made through a Model 7125S injector equipped with either a 6 µL or a 20 µL sample loop. Samples were chromatographed on HS-3 C18 (100 x 4.6 mm i.d. - part number 0254-1501) and 3x3 C18 (33 x 4.6 mm i.d. - part number 0258-0160) columns containing 3 µm C18 bonded phase particles. Short lengths of 0.007" i.d. stainless steel tubing were used for injector-to-column connections. An LC-85B variable wavelength UV detector equipped with a 1.4 µL flowcell was used for peak dispersion studies.

Amperometric detection was accomplished with the LC-4B electrochemical detector equipped with TL-5 glassy carbon electrode cell block and an Ag/AgCl reference electrode. For high-speed LC-EC, the plastic top half of the TL-5 cell block was stainless steel and also served as the auxiliary electrode. In other cases, the metal outlet

tube of the reference compartment served as the auxiliary electrode (Figure 2). Column-to-detector cell connections were made with either 0.011" i.d. Tefzel tubing (Zeus Industrial Products, Raritan, NJ) or 0.031" i.d. Teflon tubing.

Phenols and catecholamines were purchased from Sigma Chemical Company, St. Louis, MO or Aldrich Chemical Company, Milwaukee, WI. Mobile phases consisted of HPLC grade organic modifiers and deionized, distilled water. Other mobile phase additives were analytical-reagent grade. Mobile phase compositions are listed in Figure legends.

Instrumental bandwidths of the EC detector were estimated by analyzing peaks produced by 6 µL injections of phenol solution (100 ng/µL) through the chromatographic system in which the EC cell was connected directly to the injector (Figure 1). Phenol was then

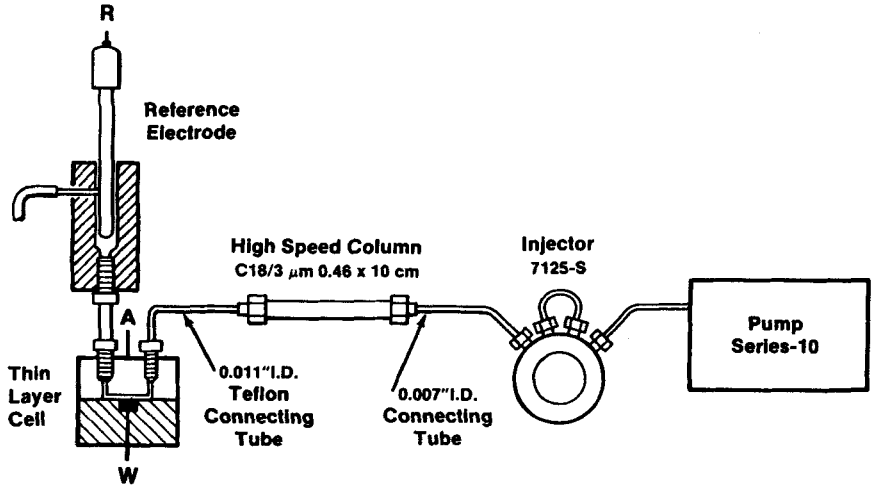

FIGURE 1
High-Speed LC-EC System.

detected at an applied potential of +1.0 V with a mobile phase of 50% acetonitrile in 0.2 M $NaClO_4$, 5 mM sodium citrate, pH 5.4. A 1-meter coil of 0.007" stainless steel tubing was placed between the injector and pump in order to produce sufficient backpressure to allow proper operation of the pulse dampener. In order to determine the upper limit of the cell volume contribution to peak dispersion, the outlet of the cell was connected to the LC-85B UV detector via a short length of 0.007" i.d. stainless steel tubing (Figure 2) with a specially made low-volume end fitting. Phenol peaks were then detected at 280 nm. By connecting the LC-85B directly to the injector, dispersion due to the system without the EC cell was estimated. Bandwidths (4 sigma) were calculated

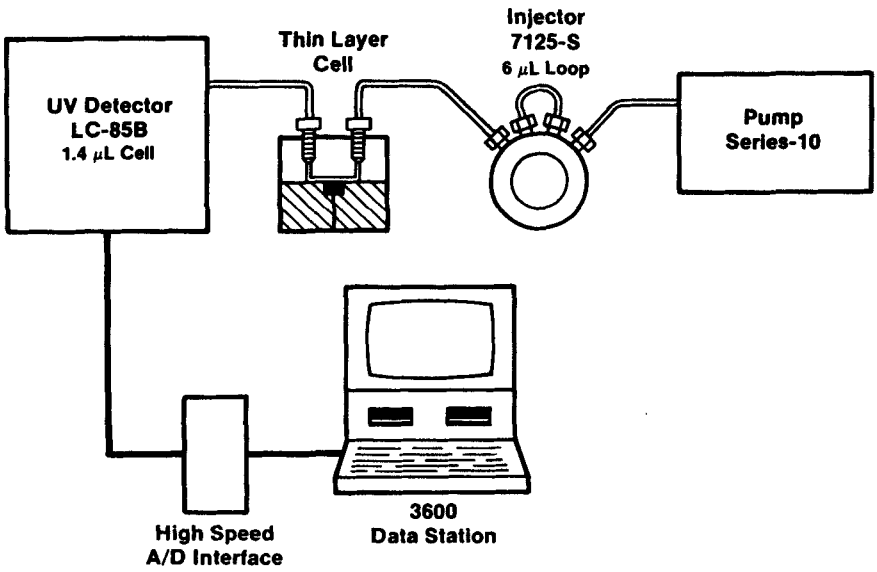

FIGURE 2

System for estimating EC flowcell hydraulics' contribution to dispersion.

by width at half-heights as measured on recorder paper and by computer determination of central moments.

Detector output signals were acquired by a Model 3600 Data Station via the Chromatography Interface (Figure 2) which is a high-speed analog to digital converter. Data acquisition and storage were controlled by software written in BASIC. Central moments of peaks were then determined as discussed by Grushka, et al. (23) using a second BASIC program. An abbreviated algorithm of this program is shown in Figure 3.

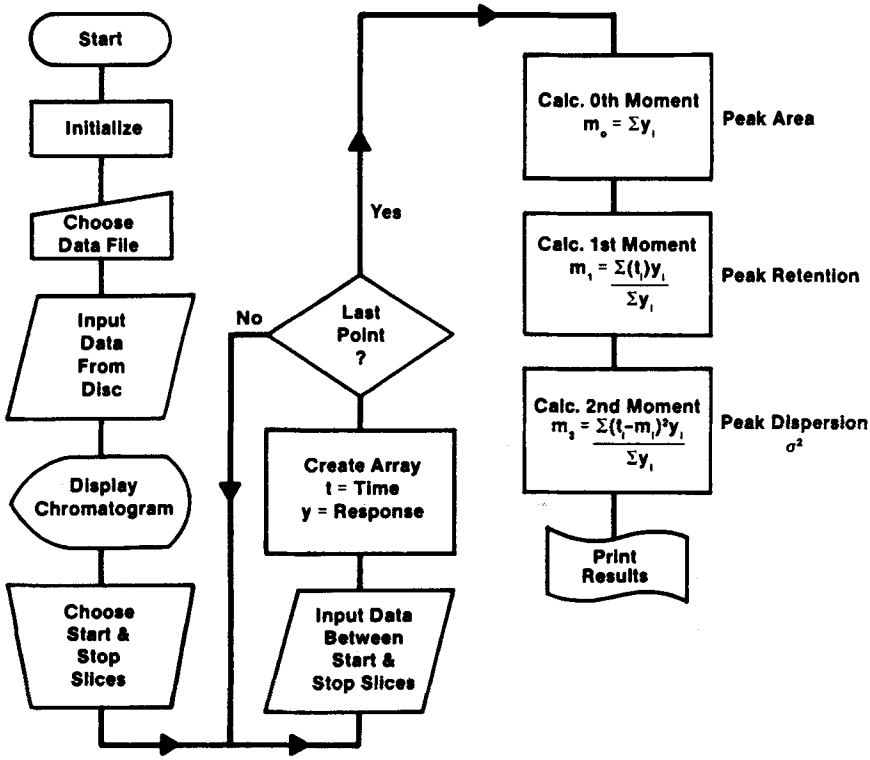

FIGURE 3

Flow chart for computerized Central Moments Determination.

RESULTS AND DISCUSSION

Instrumental Bandwidth

IBW can be defined as the band broadening of a solute zone passing through the LC system caused by all the system components except the column. IBW is determined by injecting a solute into the chromatograph with the column replaced by a zero dead volume connection (or appropriate capillary tubing directly connecting the injector and detector) and measuring the volume of the resultant eluted peak. Replacing the column with a zero-dead volume union in Figure 1 illustrates such a system. IBW is equal to four times the standard deviation (σ) of the eluting peak.

The value of σ can be estimated in many ways, analogous to the many methods of determining theoretical plate number N. The quality of the value obtained in σ estimation (or N estimation) depends on the shape of the peak and the measurement technique used. In this study we have estimated IBW using two procedures:

1. Peak width at half-height
2. Statistical moments

The half-height method allows estimation of σ and, therefore, 4 σ according to:

$$\sigma = \frac{W_h}{2.354} \qquad (1)$$

where W_h is the peak width at half-height.

This method assumes gaussian peak shape and any deviation results in an underestimation of dispersion and, therefore, an overestimation of resolving power. This method is a practical alternative since many practicing chromatographers do not have

ready access to the instrumentation and software required to apply statistical moments analysis to the general problem of estimating dispersion.

Application of the moments method provides good estimation of peak variance as illustrated by Kirkland, et al. (24) even for very asymmetrical peaks. Peak variance σ^2, the second central moment, can be expressed as (23)

$$\sigma^2 = \frac{\Sigma(t_i - m_1)^2 y_i}{\Sigma y_i} \qquad (2)$$

Where t_i = time

m_1 = the first moment

y_i = response

Figure 3 schematically illustrates the computer program for moments determination (25) using chromatographic data stored on disc. The data file is selected from disc and displayed graphically on the CRT. The beginning and end of the peak of interest is determined (either manually or automatically) and a time versus response array is created. The zeroeth, first and second moments are calculated and the results printed.

In this study, our specific aim was to estimate IBW for the high-speed LC-EC system illustrated in Figure 1 so that we could estimate the proper instrument-column match, assess the impact of improved cell design and compare the state of high-speed LC-EC with high-speed LC using UV detection.

The improvement in performance resulting from cell design modifications is very apparent by comparing Figure 4a with 4b. The

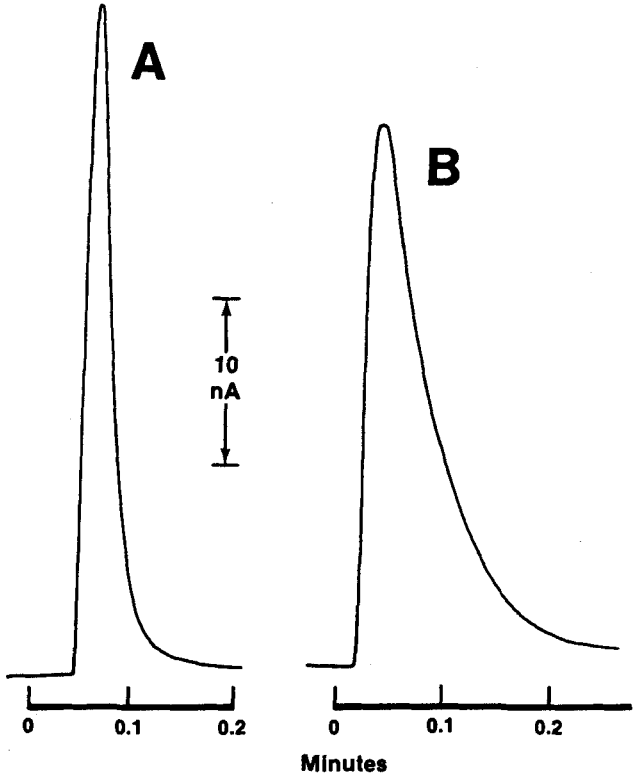

FIGURE 4

Dispersion of new versus old cell design. (A) High-speed cell 4 σ = 47 µL by W_h, 4 σ = 64 µL by moments. (B) Conventional cell, 4 σ = 113 µL by W_h, 4 σ = 164 µL by moments.

estimation of IBW by half-height decreases from 113 µL to 47 µL and by moments from 164 µL to 64 µL. For a 15,000 theoretical plate column at k' = 3, this corresponds to changes in observed N of 11257 to 14184 and 8821 to 13554 using the half-height and moments estimations of IBW, respectively. The resultant decrease in bandwidth is obvious just by looking at the width and tailing of the two peaks. It's interesting that this improvement in high-speed

LC-EC is roughly equivalent to the improvement reported for the first high-speed LC systems using UV detection relative to 'conventional' systems of the time (1-3). The practical conclusion is columns of about 100 x 4.6 mm packed with 3 μm particles are compatible with high-speed LC-EC.

This compatibility is best illustrated by the example of Table 1.

The observed efficiency ($N_{observed}$) of a 15,000 theoretical plate column (N_{col}) is compared to N_{col} with respect to % loss of N defined as

$$\% \text{ loss } N = \frac{N_{col} - N_{observed}}{N_{col}} \times 100 \qquad (3)$$

and % loss of resolution (R_s) defined as

$$\% \text{ loss } R_s = \frac{R_{s\,col} - R_{s\,observed}}{R_{s\,observed}} \times 100 \qquad (4)$$

TABLE 1

Influence of IBW on Observed Efficiency and Resolution (a)

k'	N_{col}	$N_{observed}$	% loss N	% loss R_s
0	15000	4491	70	-
1	15000	9464	37	21.0%
3	15000	13086	13	6.6%
5	15000	14085	6	3.1%
10	15000	14715	2	1.0%

(a) IBW = 50 μL, initial R_s = 1.25, 100 x 4.6 mm 3 μm column with V_o = 1 mL.

where $R_{s\ col}$ is 1.25 and

$$R_{s\ observed} = \frac{\sqrt{N_{observed}}}{\sqrt{N_{col}}} \times R_{s\ col} \qquad (5)$$

The example of Table 1 assumes IBW = 50 µL and V_o = 1.0 mL (V_o = column void volume) for a 100 x 4.6 mm column packed with 3 µm particles. At low k' (k' = capacity factor) (26), 0 to 1, 40% to 70% of the column efficiency is lost due to IBW. At k' = 3.0, only about 10% of the efficiency is lost. Of greater practical consequence is the resultant loss in R_s. Even at k' = 1, only 20% of the R_s is lost. At k' = 3, the loss in R_s is quite small, only about 6%.

It is of interest to attempt to differentiate the various instrumental contributions to band broadening. We have attempted to isolate the influence of the cell flow path (σ^2_{FP}) by placing the cell between an injector and detector of known dispersion as illustrated in Figure 2. The total EC detector variance (σ^2_{DET}) can be described as

$$\sigma^2_{DET} = \sigma^2_{FP} + \sigma^2_{ELEC} + \sigma^2_{ECP} \qquad (6)$$

where σ^2_{ELEC} = apparent dispersion resulting from the detector electronics and σ^2_{ECP} = apparent dispersion resulting from electrochemical processes (22). The σ_{FP} contribution of flow through the cell is about 5 µL by this procedure. The variance due to the flow path is estimated by subtracting the variance of the system composed of injector, connecting tubes and LC-85B from this system illustrated in Figure 2. The actual contribution of the flow path is from the detector inlet to the working electrode. σ^2_{FP} was used to estimate

the upper limit of the cell flow path volume contribution to σ^2_{DET} since the IBW data of Figure 4 led us to suspect a significant contribution from something other than flow path volume. By combining this result with the IBW of the system in Figure 1, an estimate of IBW of 'total electronic factors' 4 σ_{ELEC} = 60 μL, can be achieved. This confirms our suspicion and indicates one direction for future developments in high-speed compatible EC detectors, i.e. reduction in 'electronic factors' related dispersion. Further, more detailed studies are necessary to support this observation and should be designed to differentiate between σ^2_{ELEC} and σ^2_{ECP}.

Another experiment performed during this study further illustrates the importance of 'electronic factors' in IBW of EC cells. It is well known that positioning of the auxiliary electrode relative to the working electrode is very influential regarding detector response (22). Generally, the closer the auxiliary electrode is to the working electrode, the greater the linear range, all other factors being equal. With the new cell design, there are two convenient auxiliary electrode positions - the top half of the cell block and the outlet tube from the reference electrode (see Figure 1). Figure 5 compares IBW as determined using the two different auxiliary electrode configurations. Clearly, using the top half of the cell as the auxiliary electrode is vastly superior, presumably by minimizing the resistance and concomitantly sharpening the potential gradient.

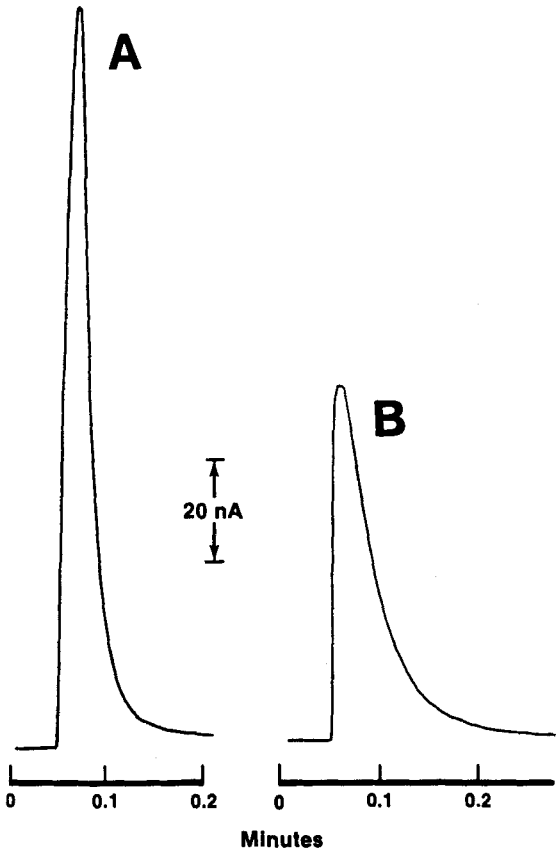

FIGURE 5

Effect of auxiliary electrode position on dispersion. (A) top half of cell as auxiliary electrode : 4 σ = 47 µL by W_h, 4 σ = 64 µL by moments. (B) Outlet tube as auxiliary electrode: 4 σ = 74 µL by W_h, 4 σ = 109 µL by moments.

Another important consideration in high-speed LC-EC is the dependence of IBW on flow rate. Over the flow rate range examined, IBW increases linear with flow rate (Figure 6). This is distinctly different from results reported for a high-speed UV detector where

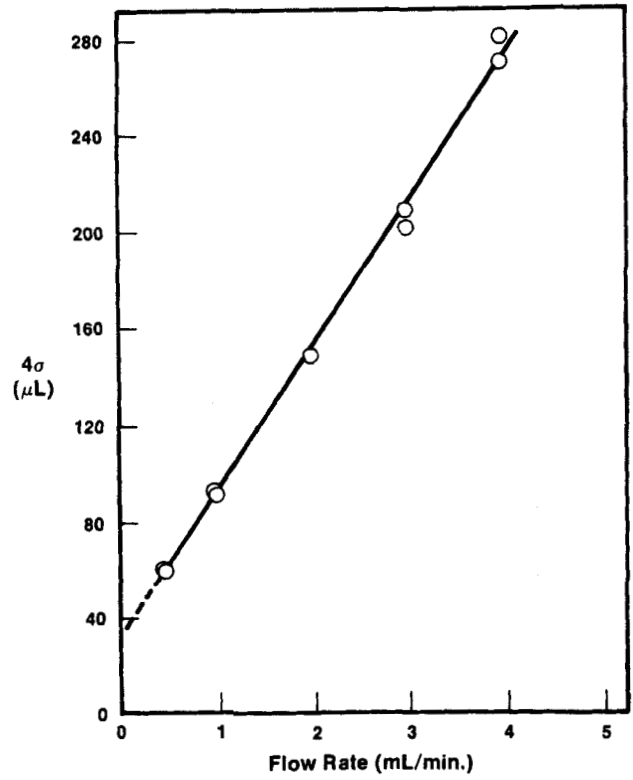

FIGURE 6

The influence of flow rate on dispersion in the high-speed EC cell.

IBW does not increase, but actually decreases slightly over the flow rate range of the present study (2). At the present time, the reasons for the different relationships between IBW and flow rate for the two different detector types are unknown. An understanding of this phenomena would be quite useful in further improving the high-speed characteristics of the EC cell.

Applications of High-Speed LC-EC

The benefits of high-speed LC-EC are best illustrated by comparison of high-speed chromatograms with 'conventional' LC-EC chromato-

grams. The separation of two important sample types, phenols and catecholamines will be used for this purpose.

The determination of phenols in environmental samples is important due to their toxicity and widespread use. Recently, Dong and DiCesare (16) have reported high-speed LC-EC separations of phenols, and Shoup and Mayer (27) have reported the determination of phenols in environmental samples. High-speed isocratic and gradient separations of phenols are demonstrated in Figures 7 and 8. A high-speed C18 column using 3 μm particles, 100 x 4.6 mm i.d. was used to achieve 3-minute isocratic and 6-minute gradient chromatograms. Similar conventional separations required over 20 minutes (28, 29).

The physiological importance and diagnostic value of catecholamine assays necessitate their optimization in terms of both speed of analysis and sensitivity. LC-EC has great potential in this respect, as evidenced by recent reviews of various protocols for the quantitation of subnanogram levels of catecholamines (30-32).

Using a mobile phase of 0.1 M formic acid containing 4% (V/V) acetonitrile, 1 M EDTA and 0.2 mM sodium octyl sulfate at a pH of 3.2 (adjusted with solid KOH) and a flow rate of 3 mL/min, the isocratic separation of DOPA, norepinephrine, epinephrine, acetaminophen and dopamine was accomplished within four minutes on a 100 x 4.6 mm i.d. high-speed 3 μm C18 column (Figure 9). This chromatogram was detected using the optimized system in which the metal cell block served as the auxiliary electrode and was connected to the column via a 10 cm length of 0.011" i.d. tubing. The suitability of this system for plasma samples will be the subject of a forthcoming paper.

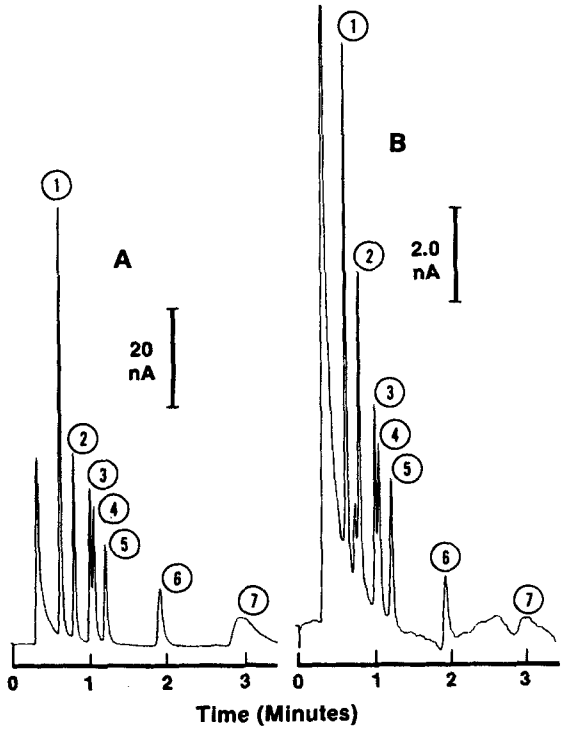

FIGURE 7

Isocratic high-speed LC-EC separation of phenols. Column: C18 - 3 μm. Pump: SERIES 4. Detector: LC-4B (+0.9 V). Flow rate: 2.5 mL/min. Mobile phase: 50% acetonitrile/50% aqueous solution of 0.2 M $NaClO_4$, 0.005 M sodium citrate (pH 5.4). Peak identification: (1) phenol, (2) o-chlorophenol, (3) 2,4-dimethylphenol, (4) 4-chloro-m-cresol, (5) 2,4 dichlorophenol, (6) 2,4,6-trichlorophenol, (7) pentachlorophenol. Amount injected in A: (1) 800 pg, (2) 700 pg, (3) 300 pg, (4) 1300 pg, (5) 800 pg, (6) 1150 pg and (7) 14000 pg. Amount injected in B is 10 times less than A.

Using the same mobile phase at a flow rate of 4 mL/min, this same separation was accomplished in one-minute with the 33 x 4.6 mm column (Figure 10). The use of the shorter column at one-fourth the analysis time of the 10 cm column resulted in a small loss of

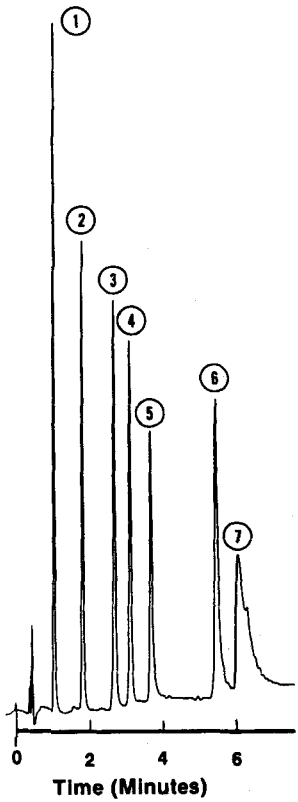

FIGURE 8

Gradient high-speed LC-EC separation of phenols. Conditions as in Figure 8 except mobile phase. Mobile phase: linear gradient 0% to 60%A in 6 minutes. A = acetonitrile, B = 30% acetonitrile/70% aqueous solution of 0.2 M $NaClO_4$, 0.005 M sodium citrate (pH 5.4).

resolution which could be compensated for by adjustment of mobile phase components (33). However, increased baseline noise observed with the short column prevented acceptable detection below 300 pg. This phenomenon is currently under investigation. It is apparent that further improvements in EC detector design, including flow

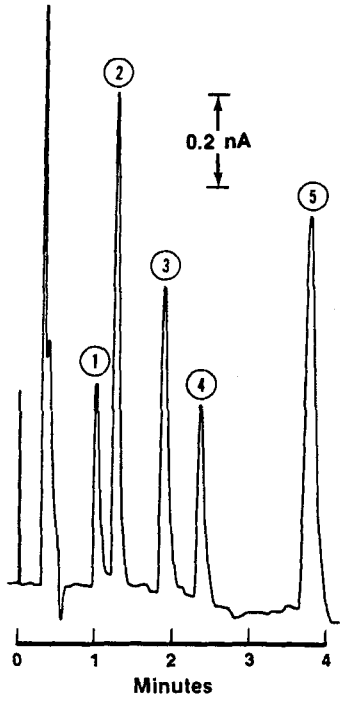

FIGURE 9

High-speed LC-EC separation of catecholamines and acetaminophen. Column: C18-3 μm, 0.46 x 10 cm. Pump: SERIES 10. Detector: LC-4B, + 600 mV. Flow rate: 3 mL/min. Mobile phase: aqueous solution of 0.1 M formic acid, 4% (V/V) acetonitrile, 0.001 M EDTA, 0.0002 M sodium octyl sulfate, adjusted to pH 3.2 with KOH. Peak identification: 300 picograms each of (1) L-dopa, (2) norepinephrine, (3) epinephrine, (4) acetaminophen, (5) dopamine.

geometry of the cell as well as electronics, are needed in order to match the performance of the shorter column.

CONCLUSIONS

The IBW of a conventional LC-EC system has been estimated at approximately 160 μL by a computerized central moments procedure. An

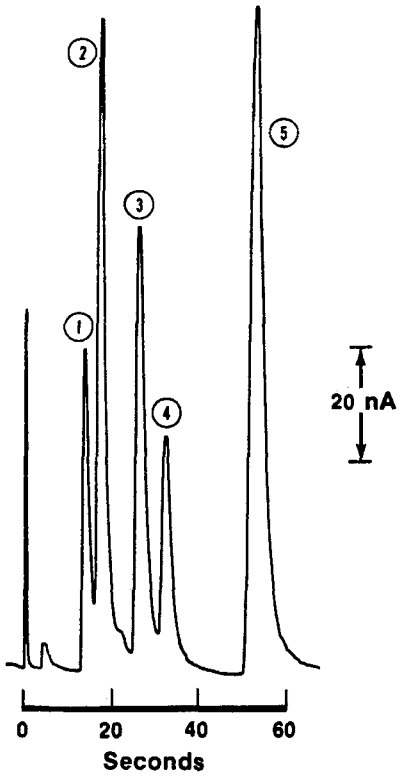

FIGURE 10

High-speed LC-EC separation of catecholamines and acetaminophen using a 3.3 cm column. Conditions as in Figure 10 except column length is 3.3 cm and flow rate is 4.0 mL/min. Peak identification: 60 ng each of (1) L-dopa, (2) norepinephrine, (3) epinephrine, (4) acetaminophen, (5) dopamine.

improved high-speed LC-EC system has an IBW of approximately 60 µL. The metal block EC cell design permitting use of 0.011" connecting tubing and proper location of the auxiliary electrode is responsible for the improved IBW characteristics. This system is compatible with high-speed 3 µm particle columns, 100 x 4.6 mm and is roughly compa-

rable to reported fluorescence and refractive index detector-based systems with respect to IBW. High-speed LC-EC has not achieved the IBW performance reported for systems using UV detection (i.e. IBW = 15 μL) (12), but may well be capable of evolving to that performance level.

REFERENCES

1. DiCesare, J.L., Dong, M.W. and Ettre, L.S., Chromatographia, 14, 257, 1981.
2. DiCesare, J.L., Dong, M.W. and Atwood, J.G., J. Chromatogr., 217, 369, 1981.
3. DiCesare, J.L., Dong, M.W. and Ettre, L.S., Introduction to High-Speed Liquid Chromatography, Perkin-Elmer, Norwalk, CT, 1981.
4. Cooke, N.H.C., Sivorinovsky, G., Archer, B.G., Paper No. 773 presented at the Pittsburgh Conference, 1982, Atlantic City, NJ.
5. Dong, M.W. and DiCesare, J.L., J. Chromatogr., 253, 159, 1982.
7. DiCesare, J.L., Dong, M.W. and Vandemark, F.L., Amer. Lab., 13, 52, 1981.
8. Dong, M.W. and DiCesare, J.L., J. Chromatogr. Sci., 20, 330, 1982.
9. Dong, M.W. and DiCesare, J.L., J. Chromatogr. Sci., 20 517, 1982.
10. Dong, M.W. and DiCesare, J.L., Food Technol., 36, 58, 1983.
11. Katz, E. and Scott, R.P.W., Submitted to J. Chromatogr.
12. DiCesare, J.L., Ind. Res. and Devel., 110, April, 1983.
13. Katz, E. and Scott, R.P.W., J. Chromatogr., 246, 191, 1982.
14. DiCesare, J.L., Personal Communication.
15. Miller, R.L., Paper No. 954 presented at the 1983 Pittsburgh Conference, Atlantic City, NJ.
16. Dong, M.E. and DiCesare, J.L., Current Separations, in press.
17. Recent Reports on Liquid Chromatography/Electrochemistry, Shoup, R.E., editor, BAS, West Lafayette, IN, 1982.

18. King, W.P. and Kissinger, P.T., Clin. Chem., 26, 1484, 1980.

19. Jacobs, W.A. and Kissinger, P.T., J. Liquid Chromatogr., 5, 881, 1982.

20. Riggin, R.M. and Howard, C.C., Anal. Chem., 51, 210, 1979.

21. Kissinger, P.T., Anal. Chem., 49, 447A, 1979.

22. Weber, S.G. and Purdy, W.C., Ind. Eng. Chem. Prod. Res. Dev., 20, 593, 1981.

23. Grushka, E., Myers, M.N., Schettler, P.D. and Giddings, J.C., Anal. Chem., 41, 889, 1969.

24. Kirkland, J.J., You, W.W., Stoklosa, H.J. and Dilks, C.H., Jr., J. Chromatogr. Sci., 15, 303, 1977.

25. DiBussolo, J.M., Unpublished Results.

26. Karger, B.L., Snyder, L.R. and Horvath, C., An Introduction to Separation Science, John Wiley and Sons, New York, 30, 1973.

27. Shoup, R.E. and Mayer, G.S., Anal. Chem., 54, 1164, 1982.

28. Realini, R.A., J. Chromatogr. Sci., 19, 124, 1981.

29. Ogan, K. and Katz, E., Anal. Chem., 53, 160, 1981.

30. Allenmark, S., J. Liquid Chromatogr., 5, 1, 1982.

31. Krstulovic, A.M., J. Chromatogr., 229, 1, 1982.

32. Holly, J.M.P. and Makin, H.L.J., Anal. Biochem., 128, 257, 1983.

33. Lammers, N., Zeeman, J. and deJong, G.J., J. HRC & C, 4, 444, 1981.