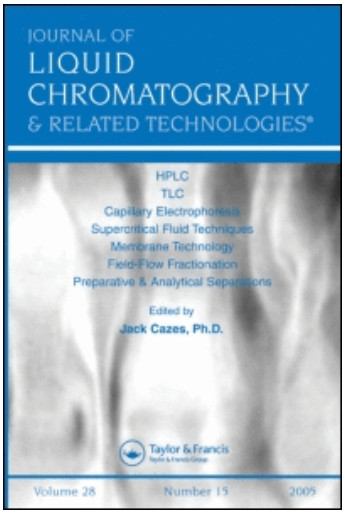

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Tetrahydrobiopterin and its Analogues in Biological Samples by Microbore Liquid Chromatography

Fuming Xie[a]; Peter T. Kissinger[a]; Osamu Niwa[b]
[a] Bioanalytical Systems, Inc., West Lafayette, IN, USA [b] NTT Basic Research Lab. NTT Corp., Kanagawa, Japan

To cite this Article Xie, Fuming , Kissinger, Peter T. and Niwa, Osamu(1997) 'Determination of Tetrahydrobiopterin and its Analogues in Biological Samples by Microbore Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 20: 6, 825 — 837
To link to this Article: DOI: 10.1080/10826079708013656
URL: http://dx.doi.org/10.1080/10826079708013656

J. LIQ. CHROM. & REL. TECHNOL., 20(6), 825-837 (1997)

DETERMINATION OF TETRAHYDROBIOPTERIN AND ITS ANALOGUES IN BIOLOGICAL SAMPLES BY MICROBORE LIQUID CHROMATOGRAPHY

Fuming Xie,[1]* Peter T. Kissinger,[1] Osamu Niwa[2]

[1]Bioanalytical Systems, Inc.
West Lafayette, IN 47906, USA

[2]NTT Basic Research Lab.
NTT Corp.
3-1 Morinosato Wakamiya
Atsugi-Shi, Kanagawa, 243-01, Japan

ABSTRACT

Tetrahydrobiopterin and seven of its analogues (neopterin, xanthopterin, biopterin, pterin, tetrahydropterin, 6-methyltetrahydropterin, and 6-methylpterin) were separated on a 1 x 150 mm C_{18} microbore column. These analytes were detected by dual-electrode amperometry and UV absorption. Both a conventional glassy carbon electrode and an interdigitated array microelectrode were used. Low fmol amounts of xanthopterin, tetrahydropterin, tetrahydrobiopterin and 6-methyl-tetrahydropterin could be determined by electrochemical detection in the oxidative mode, but pmol amounts of the other analogues were determined by electrochemical detection in the reductive mode and with UV detection.

Catecholamines and their metabolites do not interfere with the determination of tetrahydrobiopterin and its analogues in biological samples. The developed method was explored for the determination of tetrahydrobiopterin in samples of human urine and rat tissue (brain, liver, and kidney).

INTRODUCTION

The pterins are a family of nitrogen heterocyclics that are currently of interest in medicine and biology. Among them, tetrahydrobiopterin (BH_4) has been drawing the most attention because it is a key cofactor essential for phenylalanine hydroxylase which catalyzes the synthesis of neurotransmitters including dopamine, epinephrine, norepinephrine, and serotonin in the CNS.[1] BH_4 is also a cofactor for nitric oxide synthase which catalyzes the production of nitric oxide, a candidate neurotransmitter.[2] In the brain of patients with either Parkinson's[3] or Alzheimer's disease,[4] a decreased concentration of BH_4 is thought to be associated with the decreased rate of neurotransmitter synthesis. Altered excretion patterns of pterins have also been observed in patients with cancer.[5,6] Therefore, a simple, sensitive and rapid analytical method for the simultaneous determination of BH_4 and its analogues in biological samples is desirable.

Pterins in biological samples have been determined by liquid chromatography with electrochemical detection (LCEC) and/or fluorimetric detection.[7-9] These methods are satisfactory for many purposes. In this study, a microbore column was used to lower the detection limit and to make the measurement of BH_4 and its analogues in biological samples somewhat simpler than for previous methods. An interdigitated array (IDA) microelectrode based on carbon film technology was also explored for determination of the reduced pterins.

MATERIALS AND METHODS

Chemicals and Standards

(6R)-5,6,7,8-Tetrahydro-L-biopterin (BH_4), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (EP), norepinephrine (NEP), 5-hydroxy-indoleacetic acid (5-HIAA), homovanillic acid (HVA), dopamine (DA), and serotonin (5-HT) were obtained from Research Biochemicals International, Natick, MA. 5,6,7,8-Tetrahydropterin (THP), D(+)-neopterin (NEP), and (1'S,

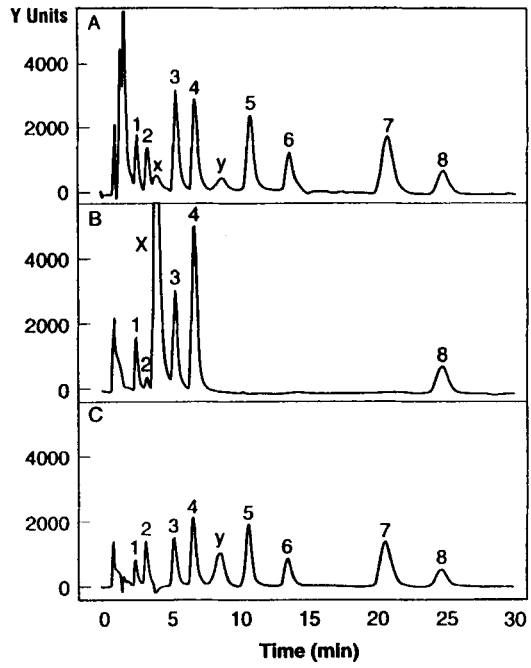

Figure 1. Chromatograms of BH_4 and its analogues using dual series glassy carbon electrodes for EC detection followed by UV detection. Microbore LC conditions: analytical column, UniJet C_{18}, 5 µm, 1 x 150 mm; mobile phase, 0.8% acetonitrile in 0.1 M phosphate buffer (pH 3.0), 4.5 mM sodium octylsulfonate, 54 µM disodium EDTA , which was maintained at 35°C; flow rate, 100 µL/min. The oven temperature containing the columns and detectors was maintained at 50°C . (A) UV detection, 220 nm; (B) the downstream EC detection, applied potential of -0.7 V vs. Ag/AgCl; (C) the upstream EC detection, applied potential of +0.75 V vs. Ag/AgCl.
1 = NEP; 2 = XANP; 3 = BP; 4 = P; 5 = THP; 6 = BH_4; 7 = M-THP; 8 = M-P; X = oxygen; Y = system peak.

2'R)- biopterin (BP) were purchased from Fluka Chemical Corp., Ronkonkoma, NY, USA. Pterin (P), 6-methylpterin (M-P) and xanthopterin (XAP) were supplied by Sigma Chemical Co., St. Louis, MO, USA. DL-6-methyl-5,6,7,8-tetrahydropterin (M-THP) was obtained from Calbiochem-Novabiochem Corp., La Jolla, CA, USA. All other chemicals were of analytical-reagent grade and were used as received.

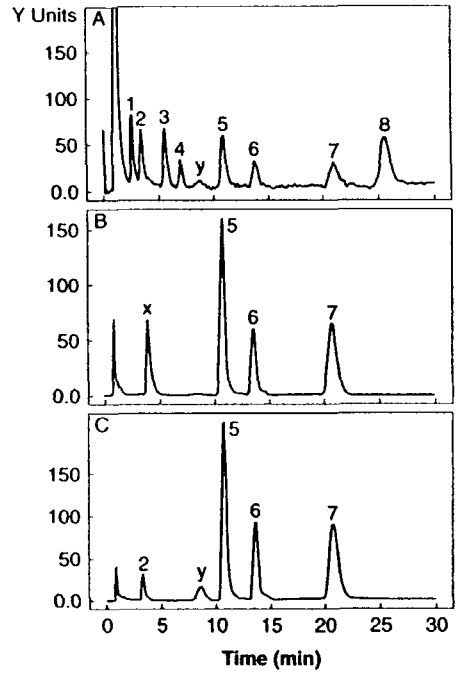

Figure 2. Chromatograms of BH_4 and its analogues using IDA dual electrodes for EC detection followed by UV detection.
Microbore LC conditions: column and mobile phase as in Figure 1.
(A) UV detection, 220 nm; (B) applied potential of -0.3 V vs. Ag/AgCl; (C) applied potential of +0.75 V vs. Ag/AgCl.
The peak assignments as in Figure 1.

Collection and Preparation of Human Urine Sample

Urine sample from a healthy volunteer was collected and immediately mixed with 10 N hydrochloric acid containing 1 mM disodium EDTA (10:1, v/v). The acidified urine sample was diluted to 1:5 (v/v) with distilled water and then filtered through a 0.2 μm membrane microfilter (MF-1 centrifugal microfilter, BAS, West Lafayette, IN, USA). An aliquot (5 μL) of the filtrate was subjected to LCEC analysis within 12 hours of storage at room temperature or within 2 weeks of storage in the dark at -20°C.

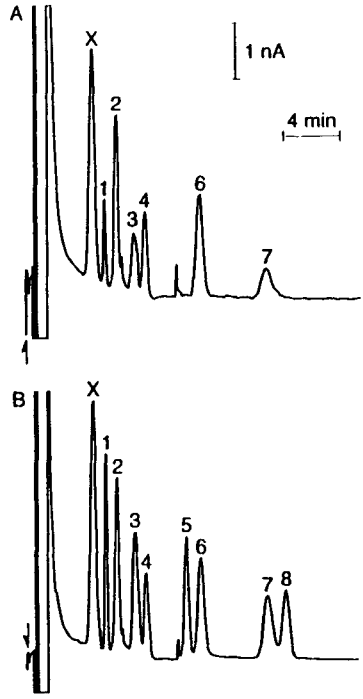

Figure 3. Chromatograms of catecholamines, their metabolites and reduced pterin derivatives. Microbore LC conditions as in Figure 1.
EC detection using dual parallel glassy carbon electrodes; (A) applied potential of +0.4 V vs. Ag/AgCl; (B) applied potential of +0.6 V vs. Ag/AgCl.
Peaks: 1 = DOPAC, 2 = THP, 3 = NE, 4 = BH_4, 5 = 5-HIAA, 6 = M-THP, 7 = EP, 8 = HVA, X = Decomposition peak from pterin standards.

Table 1

The Retention Factor (k) of Catecholamines, their Related Metabolites and BH_4 Derivatives

Cpd.:	DOPAC	THP	NEP	BH_4	5-HIAA	M-THP	EP	HVA	DA	5-HT
k value:*	5.7	6.7	8.2	9.2	12.4	13.8	19.4	21.1	60.0	>144

*The LCEC conditions as described for BH_4 and its analogues in the text.
t_0: 50 seconds.

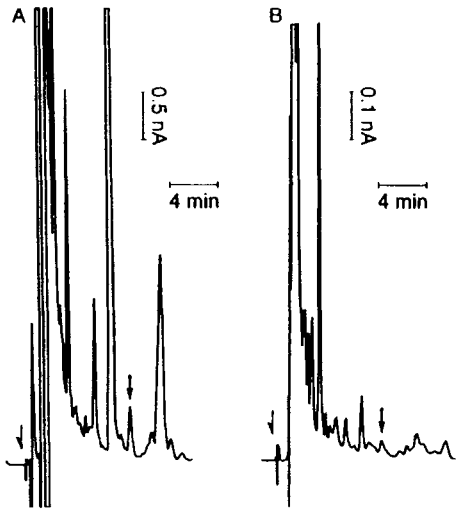

Figure 4. Chromatograms of BH_4 in urine of a healthy volunteer.
Microbore LC conditions as Figure 1.
(A) EC detection using a glassy carbon electrode, applied potential of +0.45 V vs. Ag/AgCl; (B) EC detection using an IDA electrode, applied potential of +0.4 V vs. Ag/AgCl. Arrow, BH_4

Preparation of Rat Tissue Samples

Rats were sacrificed by decapitation. The brain, liver, and kidney were quickly removed. Approximately 1 g each of the brain, liver and kidney were sliced and homogenized in 2 mL, 5 mL and 1 mL of 1 N hydrochloric acid containing 0.1 mM disodium EDTA, respectively. Following centrifugation at 1315 x g, each supernatant was filtered through a 0.2 μm membrane microfilter. A 5 μL aliquot of the filtrate was injected into the LCEC system. The samples were prepared and analyzed within 12 hours of storage at room temperature or within 2 weeks of storage in the dark at -20°C.

LCEC/LCUV

Determination of BH_4 and its analogues was performed on a BAS 200B chromatograph equipped with low dead volume micro-injector (5 μL loop, BAS), an EC detector using either a conventional glassy carbon electrode (3 mm) or a carbon IDA microelectrode (gap and width: 2 μm; cathode and

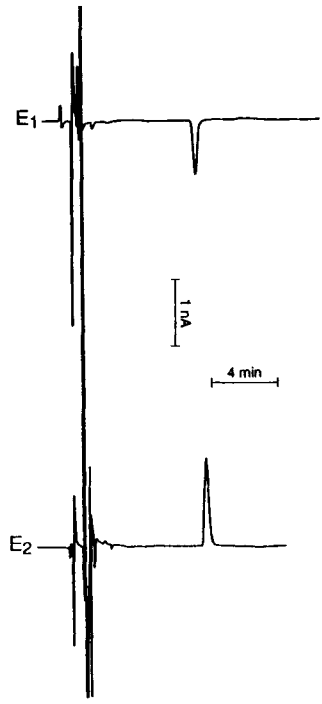

Figure 5. Chromatogram of standard BH_4.

Microbore LC conditions as Figure 1. EC detection using dual parallel glassy carbon electrodes; upper, applied potential of +0.45 V vs. Ag/AgCl; lower, applied potential of +0.6 V vs. Ag/AgCl.

anode: each 250 pairs; length: 2 mm; thickness of carbon film: 0.1 - 0.2 μ; NTT, Kanagawa, Japan) and a UV detector in series after the EC cell. The analytical column was a UniJet C_{18}, 5 μm, 1 x 150 mm (BAS); a UniJet C_{18} column, 5 μm, 1 x 100 mm (BAS) was installed before the injector to raise the overall system pressure for optimal pump performance. The oven temperature containing the columns and detectors was maintained at 50°C. The mobile phase was 0.8 % acetonitrile in 0.1 M monobasic sodium phosphate (pH 3.0), 4.5 mM sodium octylsulfonate, 54 μM disodium EDTA maintained at 35°C. The flow rate was 100 μL/min. EC detection using either dual glassy carbon or IDA electrodes (see reference 10 for the description of the IDA electrode) was at the indicated applied potentials vs. Ag/AgCl. UV detection in series after the EC cell was at 220 nm (1.2 μL flow cell with a 3 mm path length).

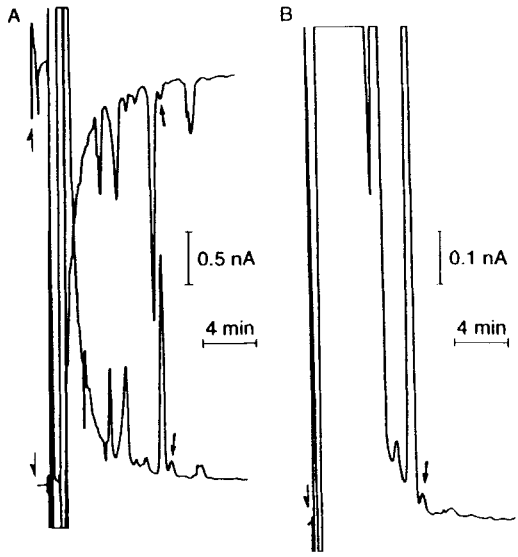

Figure 6. Chromatograms of BH_4 in rat brain sample.
Microbore LC conditions as in Figure 1.
(A) EC detection using dual parallel glassy carbon electrodes; upper, applied potential of +0.6 V vs. Ag/AgCl; lower, applied potential of +0.4 V vs. Ag/AgCl; (B) EC detection using an IDA electrode, applied potential of +0.4 V vs. Ag/AgCl. Arrow, BH_4.

RESULTS AND DISCUSSION

Initially, the separation of standards of BH_4 and its analogues was carried out on the microbore column. Various combinations of buffer solution, ion-pair and organic solvent were examined as a suitable mobile phase. The most satisfactory separation was obtained by using the mobile phase 0.8 % acetonitrile in 0.1 M monobasic sodium phosphate (pH 3.0), 4.5 mM sodium octylsulfonate, 54 μM disodium EDTA and maintaining the separation temperature at 50°C (Figure 1A). Both the oxidized and reduced forms of the pterins could be determined by EC detection corresponding to the reduction and oxidation process, respectively, at dual series glassy carbon electrodes (Figure 1B and 1C).

The IDA electrode, which was recently explored for LCEC via an joint research project by NTT, R&D Center, Kanagawa, Japan and BAS, USA,[10-13]

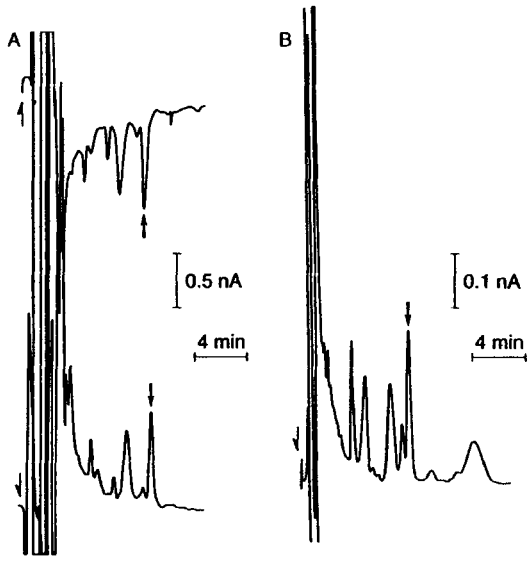

Figure 7. Chromatograms of BH_4 in rat liver sample.
Microbore LC conditions and EC conditions for A and B, respectively, as in Figure 6.

was also used for the detection of BH_4 and its analogues. BH_4 could not be detected by reductive mode downstream after it was oxidized upstream using the conventional dual series glassy carbon electrodes, but it could be detected when IDA electrode was used in the reductive mode (Figure 1B, 1C and Figure 2B, 2C).

In order to determine the pterins over a wide concentration range, we have used EC detection with dual series glassy carbon electrodes followed by UV detection. A linear response was observed for 0.1 - 100 pmoles on column for all 8 pterins using either oxidative or reductive EC detection and by UV absorbance, BH_4 exhibited a linear response from 32 fmoles on column only when using oxidative EC detection. The injected detection limit for BH_4 was 3.2 fmoles on column.

The stability of the pterins in various aqueous solutions was also examined. The reduced pterins are stable in either 1 N HCl or 1 N $HClO_4$ containing 0.1 mM disodium EDTA for 12 hours of storage at room temperature and for at least 2 weeks of storage in the dark at -20° C.

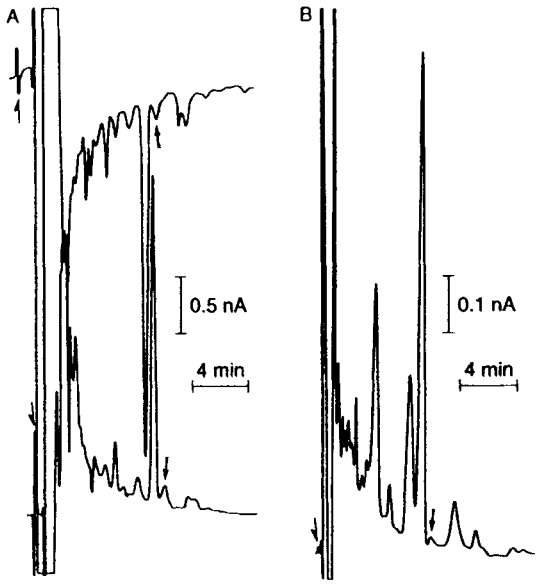

Figure 8. Chromatograms of BH_4 in rat kidney sample.
Microbore LC conditions and EC conditions for A and B, respectively, as in Figure 6.

Because the catecholamines and their metabolites are endogenous substances present *in vivo*, especially in brain, and can be detected by LCEC under the present conditions (oxidative), it is necessary to explore whether these compounds will interfere with the determination of BH_4 and its analogues. Mixtures of standard catecholamines and their metabolites were injected on this microbore LCEC system; the retention factor (k) of those compounds are shown in Table 1. All of the examined compounds possess a retention factor different from BH_4 and its analogues and can be baseline separated from them (Figure 3). The retention time of dopamine was longer than 50 minutes and serotonin was retained on column for more than 2 hours under the present conditions.

The developed method was then applied to the analysis of urine samples from a healthy volunteer. Collected urine was immediately acidified, diluted with distilled water and filtered using a centrifugal microfilter. An aliquot of the filtrate was directly injected on column. As shown in Figure 4, BH_4 in the urine sample is separated and detected without any interfering peaks. Previous studies have demonstrated that the effect of the different electrode potentials on electrochemical detection responses is characteristic of an analyte and therefore is an aid in its identification.[14-16] This approach was employed in this study for

the identification of BH_4 in the urine samples. When different applied potentials were used in a dual parallel mode for the EC detection of BH_4, different responses were obtained (Figure 5). The peak assignment of BH_4 in the urine samples was based on both its retention time and the response ratio. The identical EC detection responses of standard BH_4 and the corresponding peak in urine sample obtained by using the IDA electrode provided further support of this peak identity in human urine samples.

To further illustrate the potential utility of this method, rat brain, liver, and kidney were analyzed. After sacrifice, the tissue was sliced and homogenized in 1 N HCl containing 0.1 M disodium EDTA, centrifuged, and filtered. An aliquot of each filtrate was injected into the LCEC system without further clean-up. Typical chromatograms corresponding to the homogenized samples of rat brain, liver, and kidney are shown in Figure 6, 7, 8, respectively. Such a simple treatment of biological samples not only saves time but also decreases the risk of decomposition of the labile pterins and avoids interferences often introduced by more complicated sample preparation. Substances with longer retention times than BH_4 and its analogues (for example, dopamine and serotonin) in these biological samples made the analysis time longer but they can be easily switched out by using a column switching strategy. Normally they are too dilute to be a concern in most assays.

For the characterization of BH_4 in rat tissue, the same manner described for the identification of BH_4 in urine sample was used. Both the chromatographic behavior and the characteristic electrochemical responses obtained by using different electrode potentials compared well with those of standard BH_4.

CONCLUSION

An approach for the determination of BH_4 and its analogues using dual-electrode microbore LCEC provided excellent separation and favorable detection limits combined with a very simple treatment of biological samples.

ACKNOWLEDGMENTS

The authors thank Dr. Chester Duda for his suggestions in preparing this manuscript and Dr. Jim Gitzen and Ms. Meloney Cregor for their technical support.

REFERENCES

1. K. Tanaka, S. Kaufman, S. Milstien, Proc. Natl. Acad. Sci. USA, **86**, 5864-5867 (1989).

2. G. Werner-Felmayer, E. R. Werner, D. Fuchs, A. Hausen, G. Reibnegger, K. Schmidt, G. Weiss, H. Wachter, J. Biol. Chem., **268**, 1842-1846 (1993).

3. W. Lovenberg, R. A. Levine, D. S. Robinson, M. Ebert, A. C. Williams, D. B. Calne, Science, **204**, 624-626 (1979).

4. C. Morar, S. B. Whitburn, J. A. Blair, R. J. Leeming, G. K. Willcock, J. Neurol. Neurosurg. Psychiatry, **46**, 582 (1983).

5. H. Rokos, K. Rokos, H. Frisius, H. -J. Kirstaedter, Clin. Chim. Acta, **105**, 275-286 (1980).

6. A. Hausen, H. Wachter, J. Clin. Chem. Clin. Biochem., **20**, 593-602 (1982).

7. C. E. Lunte, P. T. Kissinger, Anal. Biochem., **129**, 377-386 (1983).

8. M. Candito, T. Nagatsu, P. Chambon, M. Chatel, J. Chromatogr., **657**, 61-66 (1994).

9. Y. Tani, T. Ohno, J. Chromatogr., **617**, 249-255 (1993).

10. Y. Iwasaki, M. Morita, Curr. Sep., **14**, 2-8 (1995).

11. O. Niwa, H. Tabei, B. P. Solomom, F. Xie, P. T. Kissinger, J. Chromatogr., **670**, 21-28 (1995).

12. O. Niwa, T. Horiuchi, M. Morita, T. Huang, P. T. Kissinger, Anal. Chim. Acta, **318**, 167-173 (1996).

13. O. Niwa, M. Morita, B. P. Solomon, P. T. Kissinger, Electroanalysis, **8**, 427-433 (1996).

14. F. Xie, H. Wang, H. Shu, J. Li, J. Jiang, J. P. Chang, Y. Hsieh, J. Chromatogr., **526**, 109-118 (1990).

15. M. Hamilton, P. T. Kissinger, Drug Metab. Dispos., **14**, 5-12 (1986).

16. A. E. Rottero, P. T. Kissinger, Biomed. Chromatogr., **2**, 24-29 (1987).

Received August 14, 1996
Accepted September 6, 1996
Manuscript 4269