



February, 1993

MF-9054

INSTRUCTION MANUAL

Amino Acid Analysis Kit

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MANUFACTURER'S NOTE

This kit, either wholly or in part, is manufactured for research purposes only. Use for medical diagnosis is not intended, implied or recommended by the manufacturer. Use for this purpose and accountability for the same rests entirely with the user.

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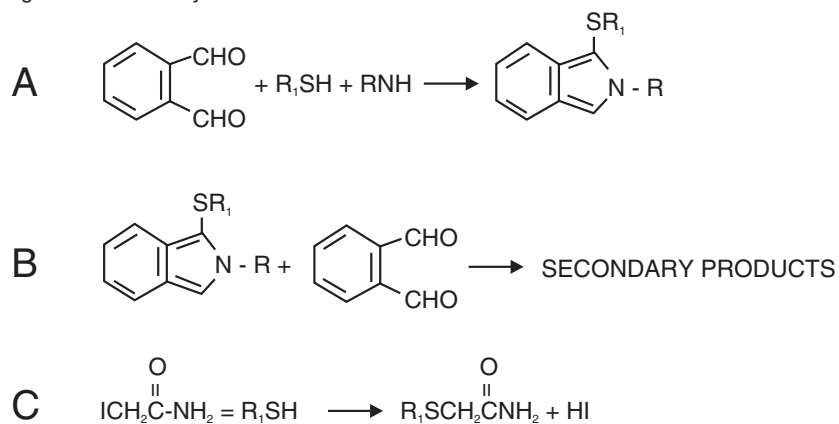
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Section 1. AN INTRODUCTION TO THE CHEMISTRY BEHIND THIS KIT

Derivatization with *o*-phthalaldehyde (OPA) is widely used for the determination of amino acids. In the presence of a thiol, OPA reacts rapidly with primary amines to form 1-alkylthioisoindoles (Figure 1.1A) in high yield. By virtue of their chromatographic and electrochemical characteristics, these derivatives are well suited to sensitive determination using LCEC. Oxidation half-wave potentials range from +0.3 to +0.6 V vs Ag/AgCl.

Figure 1.1B illustrates a common shortcoming associated with the OPA chemistry if 2-mercaptoethanol is used as the thiol. Once formed, these isoindoles are subject to degradation by multiple pathways, resulting in variable *in situ* stability (1,2). On the time scale of a determination, instability is almost entirely due to the derivative's reaction with excess OPA (1,3). This leads to the formation of secondary products which are not analytically useful. The rapid loss of parent isoindole can compromise both sensitivity and precision. Changes in thiol structure have a dramatic impact on derivative stability. BAS chemists were first to demonstrate that substitution of *tert*-butylthiol (TBT) for the more commonly used 2-mercaptoethanol or ethanethiol improves stability up to one hundred fold (1,4) and preserves the favorable redox behavior characteristic of 1-alkylthioisoindoles in general (4,5).

Figure 1.1. Formation and subsequent degradation of 1-alkylthioisoindoles.



With this stability issue resolved, the remaining concern for LCEC was the excess TBT in the sample after derivatization. The thiol is marginally detected at this potential, but its peak is still large compared to those of the amino acids. In order to scavenge the leftover TBT, iodoacetamide is added in a second step (Figure 1.1C), also performed at room temperature (6).

The 2-step BAS derivatization chemistry in this kit provides a sound basis for the detection of subpicomole quantities of amino acids. You can perform the conversion manually using pipettors, or under completely automatic control using the 2-reagent software of the BAS/CMA/200 Refrigerated Microsampler.

This manual describes three standard-bore and one microbore amino acid methods based on LCEC determination of OPA/TBT derivatized samples. These methods are designed to meet the stringent demands of modern microdialysis sampling techniques (i.e. limited sample volume combined with low analyte concentration) and provide a range of options based on user needs and priorities. They focus on various combinations of the major CNS neurotransmitter amino acids aspartic acid, glutamic acid, glycine, taurine, and γ -amino-butyric acid (GABA). Although designed specifically for use in conjunction with microdialysis, these methods are also well suited to less demanding applications such as measuring the tissue levels of these compounds.

1. W. A. Jacobs, et al., *Anal. Biochem.*, 156 (1986) 334.
2. J. F. Stobaugh, et al., *Anal. Biochem.*, 135 (1983) 495.
3. E. J. Madaj and W. A. Jacobs, *J. Org. Chem.*, 52 (1987) 3464.
4. L. A. Allison, et al., *Anal. Chem.*, 56 (1984) 1089.
5. W. A. Jacobs, *Current Separations*, 7 (1986) 39.
6. M. Goldfinger, *Abstract 434.16, Society for Neuroscience 17th Annual Meeting, New Orleans, 1987.*

Section 2. NOTICE OF APPLICABILITY

The methods described in this kit have not been validated for any *in vitro* diagnostic or clinical purposes. As with any instrumental technique, the precision and accuracy of the measurement depends on the instrumentation, the skills and knowledge of the operator, and the integrity of the sample preparation procedure. Use of these techniques for medical diagnosis and accountability for the same rests entirely with the user of this equipment.

Section 3. CONTENTS OF THE BAS AMINO ACID KITS

3.1 STANDARD-BORE KIT

The kit is specifically configured for use with an analytical system consisting of a BAS 200A Liquid Chromatograph and the BAS/CMA/200 Microsampler. The reagents are predispensed into vials compatible with the Microsampler's automatic derivatization routines. Also, the mobile phases and gradient separations described in this manual were designed using a BAS 200A with a BAS AMINO ACID II column. If you are using other types of equipment and/or columns, you may have to devise substantial changes in the operating conditions given here.

The contents of the kit are listed in Table 3.1. In addition, you can purchase the reagents, columns, and mobile phase components separately, after your first-time purchase of the complete kit. These supplies are listed in Table 3.2. By ordering BAS reagents, you can be assured that these chemicals have been tested according to the procedures outlined in this manual. The chemicals in this kit can be stored at -20 °C for the best shelf life.

If you prefer obtaining results for your scientific study as opposed to dealing with the subtle nuances of LC artifacts, use BAS EC-certified reagents.

Table 3.1. Contents of the BAS Standard-bore Amino Acid Kit (MF-8905)

QTY	DESCRIPTION
1	Manual for Kit
1	BAS AMINO ACID II cartridge column, 100 x 3.2 mm
1	Cartridge holder for above column, 100 mm
1 set	Reagent A in ready-to-use solution, set of 10 vials, 1 mL/vial
1 set	Reagent B, dry solid, set of 10 vials
1	Diluent B, 15 mL
1	Diluent A, 15 mL
4	2.5 mM Amino Acid Standard, 1 mL ampule
12	Tetrahydrofuran, EC grade, packaged under N ₂ , 230 mL
1	Material safety data sheet packet for above

Table 3.2. Reordering information for Standard-bore Kit

PART #	DESCRIPTION
MF-6199	BAS AMINO ACID II cartridge column, 100 x 3.2 mm
CF-2100	Reagent A in ready-to-use solution, set of 10 vials, 1 mL/vial Reagent B, dry solid, set of 10 vials Diluent B, 15 mL Diluent A, 15 mL 2.5 mM Amino Acid Standard, 1 mL x 4
CF-2101	Tetrahydrofuran, EC grade, packaged under N ₂ , 230 mL x 12

3.2 MICROBORE KIT

The kit is specifically configured for use with an analytical system consisting of a BAS 200A Liquid chromatograph and the CMA 200 Microsampler. The reagents are predispensed into vials compatible with the microsampler's automatic derivatization routines. Also, the mobile phases and gradient separations described in this manual were designed using a BAS-200A with a BAS SepStik microbore column and flow-splitter kit. If you are using other types of equipment and/or columns, you may have to devise substantial changes in the operating conditions given here.

The contents of the kit are listed in Table 3.3. The part numbers within the table are for purchase of replacement reagents and supplies. (The manual is not available for separate purchase.)

Table 3.3. Contents of MF-8958 Microbore Amino Acids Kit

QTY	PART	DESCRIPTION
1		Manual
1	MF-8901	3 SepStik columns, C ₈ , 5 μ m (includes fittings & gasket)
1	CF-2102	Reagents Kit
		10 vials Reagent A
		10 vials Reagent B
		1 bottle Diluent A
		1 bottle Diluent B
		4 ampules Standards
		1 pkg Material Safety Data Sheets

By purchasing BAS reagents, you can be assured that these chemicals have been tested according to the procedures outlined in this manual. The reagents should be stored at 4°C for longest shelf life. Plan on opening only one vial at a time, and using it within a few days: these reagents lose their potency when exposed to air.

Section 4. THE EQUIPMENT

4.1 SETUP AND CABLE INTERCONNECTIONS

The following components should be arranged according to Figures 4.1–4.4.

- BAS 200A ternary gradient liquid chromatograph with either LCD System Director or BASpc with System Control/Data Acquisition software. Dual amperometric detection option is strongly recommended.
- BAS/CMA/200 Refrigerated Microsampler with 2–reagent derivatization software (standard on CMA/200's with green LED on power supply) and reagent cassette (MF–5149).
- Dual pen stripchart recorder.
- Low-pressure (0–15 psi) gas regulator (BAS p/n MF–9302 or equivalent) and standard laboratory grade inert gas supply. Your regulator must be capable of 2–3 psi control. *A 100 or 300 psi regulator is not acceptable.*
- Source of cooling water if Microsampler is to operate refrigerated. This is generally unnecessary for amino acid analysis.
- For systems using the LCD System Director to control the BAS 200A, a separate integrator or workstation is recommended. The BAS product ChromGraph™ is a Windows™-based software package which emulates the functions of a dedicated integrator, but additionally provides batch reprocessing, raw and processed data storage, spreadsheet-compatible data transfer, and all the multitasking advantages of Windows™.

Figure 4.1. Equipment setup with System Director keypad as controller for BAS 200A. Data is collected at a 2-pen recorder and the ChromGraph™ Scientific Workstation (not shown).

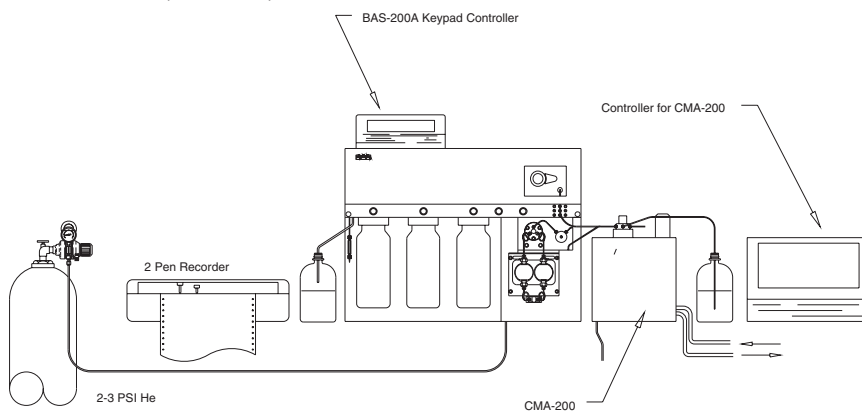


Figure 4.2. Cable diagram for Figure 4.1 (rear view).

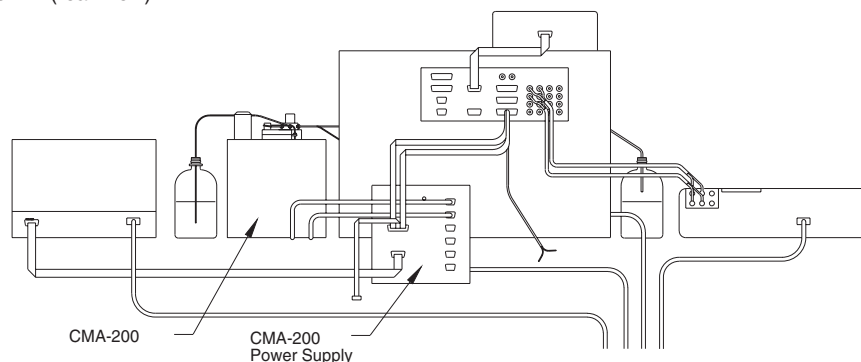


Figure 4.3. Equipment setup with BASpc System Control/Data Acquisition software. All detector data and control commands are passed over the serial interface cable.

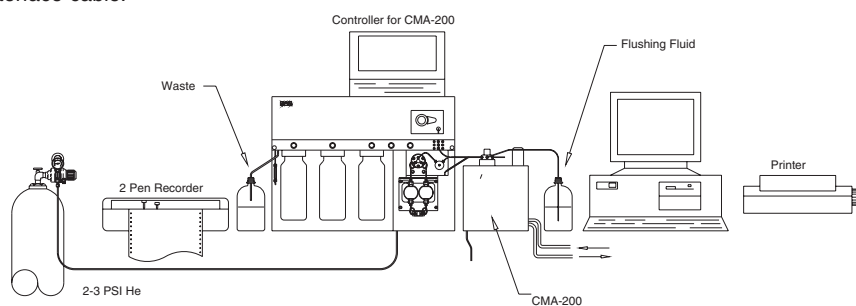
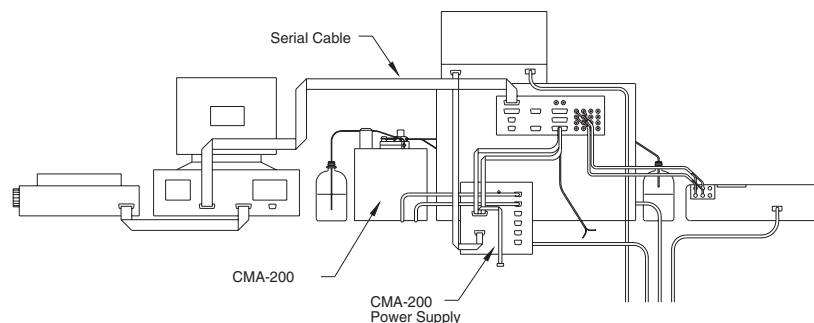


Figure 4.4. Cable diagram for Figure 4.3 (rear view).



4.2 SYSTEM FLUSHING/COLUMN WETTING PROCEDURE

The BAS 200A is rigorously cleaned and passivated at the factory. Nonetheless, BAS recommends a thorough flushing prior to use in order to assure a clean start. At the same time, the column can be properly wetted. Column wetting is absolutely essential for proper chromatographic performance.

Place ~200 mL of 100% filtered LC grade acetonitrile in bottle A and ~200 mL of 50% acetonitrile/water in bottles B and C. Open the purge valve and clear all trapped air by running a purge for 30 minutes at a 4000 psi limit and 33% A, 33% B, 34% C composition. Close the purge valve afterward. Install the column. Run the pump at 1.0 mL/min and 100% A for at least 20 minutes, then switch to 100% B for at least 20 more minutes.

Note: Solvent quality is critical to successful operation. For your reference, we use water from a 4-cartridge SYBRON Barnstead NANOpure laboratory water system. The unit constantly recirculates deionized water (from a Culligan mixed bed ion-exchanger system) through the following cartridges: D0835 Colloid/Organic Removal Cartridge, 2 each D0809 Ultrapure Deionizer Cartridges, and D0820 Organic Free Cartridge. The water output is finally filtered through a D3749 0.2 μm NANOpure Final Filter. For the assay, the term “water” shall mean water purified as described herein.

We use Baxter/Burdick and Jackson “Acetonitrile UV” with consistently good results (catalog no. 015-4). Other suppliers can also provide comparable quality solvents and purification systems.

Be aware that trace concentrations of amino acids will be found in distilled-water storage carboys and other similar locations due to bacterial contamination. Fresh is best!

Section 5. PERFORMING THE DERIVATIZATION

Prepare the reagents in the Kit as follows:

Reagent A (blue cap, contains OPA/TBT). This reagent is ready to use. Place the glass vial in position 63 (sector 4, outer row, last position). One vial should last for 3–4 days at room temperature.

Reagent B (red cap, contains iodoacetamide solid). Make this solution close to time of use and only 1 vial at a time. With a dry glass syringe and stainless disposable needle, transfer 1.0 mL of Diluent B to the glass vial of Reagent B. Keep all septa in place. When the contents are dissolved, place the vial in position 64 (sector 4, inner row, last position).

5.1 HANDLING TBT AND REAGENT A

Like all thiols, *tert*-butylthiol has a strong, characteristic, and quite unpleasant odor. TBT is contained in Reagent A. A few simple precautions can make its use pose very little trouble, however:

- Always handle the pure thiol and its concentrated solutions in a well exhausted fume hood. We advise that customers not purchase pure TBT liquid, but only “Reagent A” alone.
- Store reagents in tightly capped bottles with teflon-lined caps when not in use. Open exposure to the atmosphere permits the thiol to escape and/or oxidize.
- Small amounts of the thiol can be conveniently destroyed with household laundry bleach. (see, e.g., *Prudent Practices for Disposal of Chemicals from Laboratories*, National Academy Press, Washington D.C., 1983, pp 64–65)
- Soak all glassware, pipet tips, etc. with 10% bleach (V/V) solution to remove traces of thiol odor prior to normal washing. We keep a small plastic bucket of solution near our work area and discard everything there first.
- The working reagent can be used outside the fume hood provided it is not left open longer than necessary for pipetting. The odor is very faint if the automated derivatization procedure is used.

5.2 CMA/200 AUTOMATED DERIVATIZATION PROTOCOL

Newer CMA/200's (green LED on power supply): set up the program as shown in Figure 5.1.

Older CMA/200's (red LED): Use CMA/200 2–Reagent Software, version 1.11 (8–23–89) or later. Install according to the instructions supplied with the software. Assign the values shown in Figure 5.2 while in STANDBY, being aware that cursor movement is slow and in one direction only. Also, press F3 (Setup) and be sure all the other parameters are entered correctly for your particular microsampler.

Figure 5.1. Parameters for newer CMA-200 Microsamplers.

CMA 200

CMA/200 Left System EDIT (Uer 1.20, 920421) - 9:35:50

METHOD: 9 NAME: AMINO ACIDS FIRST SAMPLE: 1 NUMBER OF SAMPLES: 15 INJECTION VOLUME: 10.0 µL INJECTIONS PER SAMPLE: 1 ANALYSIS TIME: 25 min 0 sec STANDARD INJECTIONS / CALIBRATION: 0 REAGENT VOLUME: 2.0 µL MIX FLOW: 2000 µL/min MIX: 2 times 10 µL using LIQUID REACTION TIME: 0 sec	*** STATUS INFORMATION (Left) *** METHOD: 0 SYSTEM A: 0 -0 TIME: 7 min 23 sec TEMPERATURE CONTROL OFF 21 °C PRESENT OPERATION: STANDBY
System Method Help leading, Run parameters, Calibration, Reagent, Setup, Optional or System B	Setup Edit TEMPERATURE: 30 °C FLUSH FLOW: 2000 µL/min FLUSH VOLUME: 1000 µL < 0.9 cycles> ASPIRATE FLOW: 200 µL/min DISPENSE FLOW: 200 µL/min AIR GAP: 2.0 µL WAIT IN LOOP: 0 min 0 sec NEEDLE VOLUME: 14.9 µL VALVE VOLUME: 0.4 µL LOOP VOLUME A: 19.6 µL SAFETY VOLUME: 0.3 µL VALVE TO VALVE VOLUME: 100 µL SMALL SYRINGE: 100 µL LARGE SYRINGE: 1000 µL SEPTA ON VIALS: Y

CMA 200

CMA/200 Left System EDIT (Uer 1.20, 920421) - 9:37:07

METHOD: 9 CHAIN TO: 10 EXTRA VALVE CONTROL: OFF EXTRA REAGENT VOLUME: 2.0 µL PREFLUSH VOLUME: 0 µL < 0.0 cycles> MIX FLOW: 2000 µL/min MIX: 2 times 10 µL using LIQUID REACTION TIME: 120 sec SMALLEST REAGENT AIR GAP: 0.5 µL ALLOW AIR TO BE INJECTED: N	*** STATUS INFORMATION (Left) *** METHOD: 0 SYSTEM A: 0 -0 TIME: 8 min 39 sec TEMPERATURE CONTROL OFF 21 °C PRESENT OPERATION: STANDBY
Option Edit	Setup Edit TEMPERATURE: 30 °C FLUSH FLOW: 2000 µL/min FLUSH VOLUME: 1000 µL < 0.9 cycles> ASPIRATE FLOW: 200 µL/min DISPENSE FLOW: 200 µL/min AIR GAP: 2.0 µL WAIT IN LOOP: 0 min 0 sec NEEDLE VOLUME: 14.9 µL VALVE VOLUME: 0.4 µL LOOP VOLUME A: 19.6 µL SAFETY VOLUME: 0.3 µL VALVE TO VALVE VOLUME: 100 µL SMALL SYRINGE: 100 µL LARGE SYRINGE: 1000 µL SEPTA ON VIALS: Y

Press <space> to select External or Internal extra valve control

Figure 5.2. Parameters for older CMA-200 Microsamplers.

```

***** CMA200/MICROSAMPLER 2 REAGENTS PROGRAM *****

  *** STATUS INFORMATION ***
  SAMPLE:  INJECTION:  POS:
  TIME:    2 min 46 sec
  TEMPERATURE CONTROL OFF 33 °C
  PRESENT OPERATION:  STANDBY

METHOD NR.: 1  CHAIN TO: 0      REAGENT-A VOLUME: 2.0 µL
METHOD NAME: amino acid 1      MIXFLOW: 2000 µL/min
                                MIX: 2 TIMES: 10.0 µL
                                MIX WITH: LIQUID
                                REACTION TIME: 0 sec
FIRST SAMPLE: 1                REAGENT-B VOLUME: 2.0 µL
NUMBER OF SAMPLES: 5           MIXFLOW: 2000 µL/min
INJECTION VOLUME: 10.0 µL      MIX: 2 TIMES: 10.0 µL
INJECTIONS/SAMPLE: 1          MIX WITH: LIQUID
ANALYSIS TIME: 24 min 0 sec    REACTION TIME: 120 sec
REAGENTS (0,1 or 2): 2

***** CMA200/MICROSAMPLER 2 REAGENTS PROGRAM *****

  *** STATUS INFORMATION ***
  SAMPLE:  INJECTION:  POS:
  TIME:    3 min 49 sec
  TEMPERATURE CONTROL OFF 33 °C
  PRESENT OPERATION:  STANDBY

TEMPERATURE: 30 °C              AIR GAP: 2.0 µL
SMALL SYRINGE: 100 µL           WAIT IN LOOP: 0 sec
LARGE SYRINGE: 1000 µL          NEEDLE VOLUME: 14.3 µL
FLUSH FLOW: 2500 µL/min         LOOP VOLUME: 21.2 µL
FLUSH VOLUME: 1000 µL          SAFETY VOLUME: 0.3 µL
ASPIRATE FLOW: 100 µL/min       VALVE TO VALVE VOLUME: 100 µL
DISPENSE FLOW: 100 µL/min      SEPTA ON VIAL: YES

```

Make sure the BAS 200A is placed in SYNCHRONIZED RUNS mode and NOT configured for AUTORUNS.

Place Reagent A (OPA/TBT) in position 63, and Reagent B (IAA/MeOH) in position 64. Use water with 10% methanol as the flushing solution for the autosampler, and keep this solution covered from dandruff, lab dust, etc. Make this flush solution fresh each morning, and filter it to remove particulates and dissolved gasses.

Samples are loaded in the carousel according to the FIRST SAMPLE parameter and the sequence started by pressing START.

5.3 MANUAL DERIVATIZATION PROTOCOL

Use a good-quality 20 μL variable-volume pipettor or a 10 μL syringe. Use fresh tips to avoid cross-contamination.

- Isoindole Formation: Add 2 μL of the Reagent A to 10 μL of amino acid solution (standard or dialysate). Mix and wait 2 minutes.
- Thiol Scavenging: After the initial 2 minute reaction interval, add 2 μL of reconstituted Reagent B. Mix and wait 4 minutes (total reaction = 6 minutes) prior to injection.
- Inject 10 μL manually. You should use at least a 20 μL loop in the injection valve for a 10 μL injection, or else some sample could be lost via the overflow tubes.

5.4 DERIVATIZATION NOTES

- The reaction pH is critical for proper derivatization. The final pH must be at or above 8.0–8.5. If the samples being processed contain high acid concentrations or are strongly buffered below the desired pH, they may need to be neutralized prior to derivatizing. Improper pH control is the most common problem encountered with this chemistry.
- Test your sample's buffering capacity by adding 2 volumes of the Borate/Methanol Diluent A to 10 volumes of sample, and measure the pH. If the pH is below 8.2, use less acid or a weaker buffer concentration, or add 1–2 volumes more of the Borate/Methanol Diluent A. Retest until okay.
- The thiol scavenging step is strongly recommended regardless of the amino acids of interest, particularly for low-level analytes. Iodoacetamide alkylates the thiol, rendering it electro-inactive at the selected operating potential.
- Timing is not absolutely critical because of the excellent *in situ* stability of the OPA/TBT derivatives. However, reasonable attention to reaction timing is recommended.
- These derivatization conditions should be suitable for virtually all brain microdialysis samples. If the volume of your dialysates is different from 10 μL , adjust the volumes of Reagent A and B in direct proportion. For processing of more-concentrated solutions (i.e., tissue homogenates) it may be necessary to modify the proportions.
- Histamine is usually the first amino acid to show a decrease in linear dynamic range if thiol loss in Reagent A has occurred. Correct this by placing a fresh vial of Reagent A in the carousel and try again.
- Thiol scavenging (step 2) is impeded if the sample is in methanol or non-polar solvents. An appropriate blank should be aqueous.
- For the parameters listed in Figure 5.1, approximately 2 min. 30 sec. elapses between the dispensing of A into the sample tube and the dispensing of B. Similarly, about 3 min. 40 sec. elapses between the dispensing of B and the time of injection. These values are larger than the listed "reaction times" due to flushing and pipetting operations.

Section 6. CALIBRATION

This kit includes a standard solution of 20 amino acids in 0.1 M HCl. Table 6.1 lists the compounds and their concentrations. The listed concentrations are accurate to within $\pm 1\%$. These can be diluted by 25x in water to achieve a nominal 100 μM working solution, and further diluted to 0.5-50 μM to arrive at the appropriate calibration standards for a dialysate assay. For the 100 μM standard, it is best to remove 100 μL from the ampule and dilute it with 2.4 mL H_2O . Save the rest in another container at $-20\text{ }^\circ\text{C}$ for future use.

Periodically insert these solutions as external standards in the carousel. Use at least 3 different concentrations and generate a linearity curve by least-squares curve fitting. Interspersing a quality-control standard (i.e., 1 for every 10 unknowns) is also good practice.

Table 6.1. Amino Acid Standard Solution and Nominal Concentrations

L-Alanine	2.5 mM
4-Aminobutyric Acid	2.5
L-Arginine	2.5
L-Asparagine	2.5
L-Aspartic Acid	2.5
L-Glutamic Acid	2.5
L-Glutamine	2.5
Glycine	2.5
L-Histidine	2.5
L-Isoleucine	2.5
L-Leucine	2.5
L-Lysine	2.5
L-Methionine	2.5
L-Phenylalanine	2.5
L-Serine	2.5
Taurine	2.5
L-Threonine	2.5
L-Tryptophan	2.5
L-Tyrosine	2.5
L-Valine	2.5

Please remember that the ratio of reagents to sample is determined by the concentrations of amino acids in the sample. If you have high concentrations, the amount of reagent may not be sufficient to derivatize all of the sample. Do a linearity study to be sure of the useful range for your samples.

Section 7. PRESERVING DIALYSATES FOR AMINO ACID ANALYSIS

Due to interference with the utilization of the reagent, some commonly used recipes for stabilizing biogenic amines are not advised for amino acids. Glutathione/HClO₄, for example, is too acidic for the borate to compensate and also provides an amino group (which consumes reagent) and another thiol.

At this stage, we believe it is best to simply assay dialysates immediately upon collection, or to freeze them at -80 °C in septum-capped autosampler vials until the day of analysis. Keep the tubes upright so that the small sample stays in the bottom of the tube, or centrifuge upon thawing.

Collection of sample in a CMA/170 Fraction Collector (MF-5146) will ensure that the samples are in the same vials and cassette used in a CMA/200 Autosampler. A series of four cassettes can be stored in a covered case (MF-5157) for freezing/thawing. The CMA/170 will also collect samples in sealed vials, retarding evaporative loss. These sealed vials will also transfer directly to the autosampler.

Section 8. OBTAINING CLEAN BLANKS

You should not underestimate this problem. Amino acids are ubiquitous. The prevalent peaks in a sample contaminated by skin, dandruff, lab dust, etc. are due to serine, glycine, and alanine. Watch for this problem if you are measuring trace amounts.

If your blank runs show variable peak heights, the probable cause is contaminated sample vials. To clean the vials and septa:

- Remove the septa from the crimp rings before use.
- Soak the septa and vials in concentrated nitric acid overnight.
- Soak in dilute HCl for 4 hours.
- Rinse with water prepared fresh from distillation of alkaline potassium permanganate solution.
- Loosely wrap the parts in aluminum foil to avoid recontamination.
- Dry in an ordinary glassware oven.
- Store inside the foil until use.

Watch for finger contact and dust exposure.

Section 9. METHOD I – PUTATIVE AMINO ACID NEUROTRANSMITTERS

9.1 DESCRIPTION

This gradient elution method allows for the simultaneous monitoring of all five major neurotransmitter amino acids: aspartate, glutamate, glycine, taurine, and γ -amino butyric acid (GABA). By default, it also allows for determination of asparagine, histidine, glutamine, serine, threonine, arginine, tyrosine, alanine, and β -alanine. Amino acids more hydrophobic than GABA (starting with methionine/valine) are stripped during the column wash cycle. The gradient can be extended to obtain resolution of all common amino acids if desired. Total run time for this method (injection to injection) is about 30 minutes.

9.2 SYSTEM MODIFICATIONS

The system, system preparation, reagents, and derivatization protocol are as described in the earlier sections of this manual.

Make sure that the pulse damper is removed from the BAS-200A and that the bypass tube (from pressure transducer to purge valve) is installed in its place.

9.3 MOBILE PHASE PREPARATION

Make up 2 x 1 L of 0.1 M sodium acetate buffer, one liter at pH 6.00 \pm 0.02 (optional, see note 1 on page 18) and the other at pH 6.80 \pm 0.02, as follows:

For 1 liter, add 5.75 mL glacial acetic acid to ~900 mL water and adjust to the proper pH, first with 6 M sodium hydroxide, then 0.1 M sodium hydroxide. Use of dilute base for the final adjustments will prevent overshooting the desired pH. However, small errors can be corrected by using dilute acetic acid with no ill effects on method performance.

Note: The pH is critical. Calibrate your pH meter according to the manufacturer's instructions just prior to titrating the acetate buffers to pH 6.00 and 6.80.

Dilute to 1.0 L with water. Filter buffer through a 0.2 μ m nylon membrane.

Then make up the A, B, and C solutions. Use graduated cylinders and not volumetric flasks.

Mobile Phase A (optional, see note 1 on page 18)

- 1000 mL of pH 6.00 acetate buffer
- 163 mL of HPLC grade acetonitrile
- 29 mL of HPLC/UV grade tetrahydrofuran (preservative free)

Mobile Phase B

- 839 mL of pH 6.80 acetate buffer
- 137 mL of HPLC grade acetonitrile
- 24 mL of HPLC/UV grade tetrahydrofuran (preservative free)

Mobile Phase C

- 64 mL of pH 6.80 acetate buffer
- 191 mL of HPLC grade acetonitrile
- 170 mL of HPLC/UV grade tetrahydrofuran (preservative free)

Note: High quality tetrahydrofuran is essential. The major contributor to baseline drift in this method is the THF used in the C mobile phase. In this kit, BAS supplies a high quality material which is lot tested and “EC-certified” for LCEC use.

Do not refilter solutions A, B, or C. Place the mobile phases in their respective reservoirs and sparge for 5–10 minutes with inert gas. Close the exhaust valve and pressurize the reservoirs at 2–3 psi. Warm all bottles to 35 °C and set the oven to 40 °C. Run a 20 minute purge at 33% A, 33% B, 34% C against a 3000 psi pressure limit to flush out residual acetonitrile/water. Equilibrate the column for start up. Connect the column outlet to the cell and assemble the working and reference electrodes.

Note: After adding THF, it is important to begin sparging with inert gas immediately, in order to displace oxygen. Don't heat first, since this will aggravate baseline drift. See section 9.9, page 22.

9.4 BAS 200A METHOD FILE

PUMP FILE

High Pressure Limit	4000 psi
Low Pressure Limit	100 psi
Flow Rate	1.0 mL/min
Synchronized Runs	Yes
Autoruns	No

GRADIENT PROGRAM (see note 1)

Time (min)	%A	%B	%C
0.0	100	0	0
0.1	0	100	0
16.0	0	85	15
18.0	0	85	15
19.0	0	10	90
20.0	0	10	90
20.1	100	0	0
30.0*	100	0	0

*When 30.0 min has elapsed, the system is reequilibrated and the next injection may occur. It may be possible to shorten this time somewhat, but retention time precision should be verified before doing so. The BAS 200A will return to an EQUILIBRATING mode until the CMA/200 Microsampler's ANALYSIS TIME has elapsed and the next sample has been derivatized and loaded into the loop.

EC DETECTOR FILE

Number of Electrodes	2 Glassy Carbon
Orientation of Flowstream	Series/W1–upstream W2–downstream

DETECTOR PROGRAM

Time	LCEC	POT	GAIN	FLT	SIGN	RCDR
0.0	1	+700	100 nA	0.1	-	0
	2	+700	200 nA	0.1	+	0
30.0	1	+700	100 nA	0.1	-	0
	2	+700	200 nA	0.1	+	0

TEMPERATURE FILE

Oven	40°C
Cell	20°C
All Bottles	35°C

TIMED EVENTS FILE

Time	Event 1*	Event 2	Event 3	Event 4
Default	0	0	0	0
0.0	1	1	0	0
0.1	0	0	0	0

*When a CMA/200 Microsampler is used, this Event is reserved for BAS 200A↔CMA/200 communication.

Note 1: The lower pH of mobile phase A is designed to make Asp and Glu elute later. If this is not a problem with your system, omit mobile phase A. Reprogram the gradient to pump 100% B instead of 100% A. Or the B mobile phase may be put into bottle A and the gradient program modified accordingly.

9.5 CMA/200 METHOD FILE

Use the parameters listed in “CMA/200 Derivatization Protocol,” section 5.2, page 9.

Press FLUSH a few times to thoroughly purge the Microsampler. There should be no air in either flushing syringe. Consult the CMA/200 manual for advice.

9.6 FIRST RUNS

Equilibrate the BAS 200A's file, and set up 5 “reagent blank” runs to cycle through the gradient. This is accomplished by:

- Loading 5 septum-capped vials (filled with 10 μ L of water).
FIRST SAMPLE: 1
NO. OF SAMPLES: 5
INJECTIONS/SAMPLE: 1
- Ensuring that the BAS 200A control software says EQUILIBRATING.
- Pressing START on the CMA/200's controller.

During this time detector background will stabilize, ultimately reaching a value less than 10–15 nA.

Now dilute the supplied 2.5 mM amino acid standard 400-fold with H₂O and use 10 μ L aliquots as samples 6–10. Repeat the above sequence.

The most stable and drift free performance is obtained with repetitive runs at equally spaced time intervals. Long intervals at low solvent strength may result in on-column concentration of impurities that will be stripped off during one or two subsequent runs. This stripping may result in poor baseline quality as well as discrete interfering peaks.

The total change in background current over a “normal, good” gradient should be 1–2 nA. Should you see this change increasing significantly, or consistently being in the 20–100 nA range from the start, a probable cause is the tetrahydrofuran. See section 9.9, Special Problems, page 22.

9.7 TYPICAL RESULTS

The chromatogram in Figure 9.1(A) can be used as a reference for judging your separation. This run was produced via the two-step derivatization (isoindole formation/thiol scavenging) of 10 μ L aliquots of a 6.25 μ M amino acid standard, a 400-fold dilution of the standard solution supplied with the kit. Each peak in this chromatogram corresponds to ~45 pmole. Minor retention variations are to be expected but profiles should be quite similar to this.

Removal of thiol interference by iodoacetamide is illustrated in Figure 9.1(B) which shows a chromatogram from a 6.25 μ M standard prepared without the second reaction step. Due to the elution position of the major thiol peak in the gap between threonine and alanine, quantitative removal of thiol is not imperative. Depending on the amino acids of interest, thiol scavenging might be considered unnecessary. It is, however, strongly recommended since this will produce the cleanest, most stable baselines. Note also in Figure 9.1(B) the secondary thiol peak that appears as a shoulder on glutamate and is removed by the scavenging step.

Figure 9.2 shows chromatograms from a derivatized dialysate (rat striatum). Dialysate samples present a special difficulty due to the widely varying concentrations encountered, particularly between different amino acids. For example, GABA concentrations in recovered dialysates are typically 0.5 μ M or less while glutamine may range as high as 150–200 μ M (Table 9.1). This makes the acquisition of useful data for all amino acids at a single gain setting extremely difficult, if not impossible.

Table 9.1. Typical Amino Acid Concentrations. Homogenate values based on a 1:10 (w/v) dilution factor.

Compound	Tissue $\mu\text{mole/gm}$	Tissue Homogenate μM	Dialysate μM
Aspartate	1–2	100–200	0.2–1
Glutamate	3–12	300–1200	1–4
Glutamine	2–8	200–800	20–300
Glycine	1–3	100–300	3–8
Taurine	2–8	200–800	2–10
GABA	1–10	100–1000	0–0.7

As seen in Figure 9.2(A), run at a gain of 200 nA, the glutamine peak is offscale. Taurine, glycine, and glutamate are moderate, aspartate is small and GABA is very small. Gain programming via the BAS 200A can help overcome this problem since it allows discrete time intervals to be run at different gains without operator intervention. This strategy is viable if adequate “free space” between peaks is available to allow changes and the concurrent baseline rezeroing. Ideally, there should be at least a 60-90 second window available for this transition. Otherwise, event timing becomes extremely critical and minor drifts in retention time will dictate frequent reoptimization. An alternative to gain programming is dual electrode operation, which allows simultaneous detection at two gains. Figure 9.2(B) is from the same sample as Figure 9.2(A), running a second EC channel at a gain of 50 nA. In this trace the GABA peak is much larger.

Dual EC operation can be conducted in either series or parallel arrangements. In the example shown, the two electrodes were oriented in series with the high gain (50 nA) electrode upstream and the low gain electrode (200 nA) downstream. Both electrodes were poised at the specified potential of +0.7 V vs Ag/AgCl. Upstream operation at high gain was selected because this electrode position will typically be 10–20% more sensitive than the downstream electrode. If used in parallel the choice is entirely arbitrary. In either case, each EC channel must be independently calibrated.

Figure 9.1. Chromatogram of 6.25 μM amino acid standard mixture, 45 pmole each. (A) Two-step derivatization per this manual, (B) same sample, but with methanol substituted for Reagent B. Note the small peak interfering with GLU (*) and also the TBT (\ddagger) peak between THR and ALA.

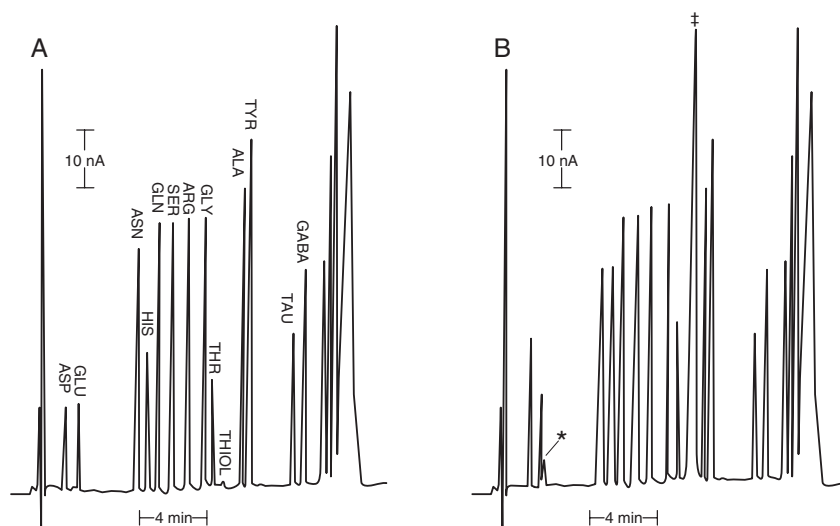
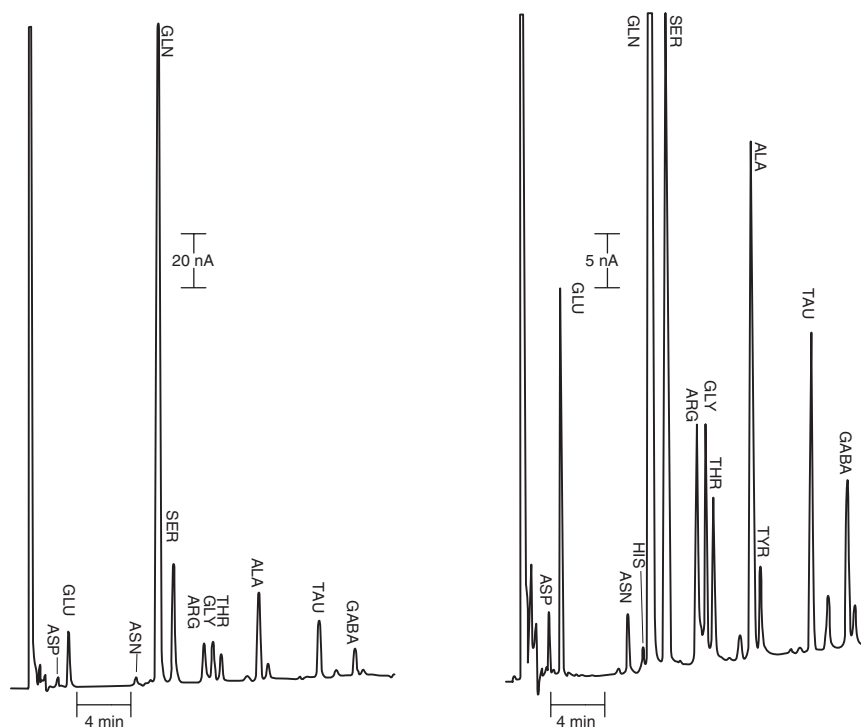


Figure 9.2. (A) Derivatized rat caudate dialysate. W1, downstream, @ 200 nAfs. (B) Same as A, except W2, upstream, @ 50 nAfs.



9.8 SEPARATION ADJUSTMENT

The major parameters controlling the separation are pH and solvent composition. By virtue of their free carboxyl moieties, all of the amino acid derivatives exhibit strong retention vs. pH dependence. Lowering the pH results in a general increase in retention due to the greater hydrophobicity of the protonated forms. Due to the differences in pK_a s and relative hydrophobicities of protonated and unprotonated forms, the specific pH window over which retention time (or the capacity factor) varies as a function of pH differs for each derivative. As structured in this method, the bulk of the separation takes place at pH 6.80, following start-up at 6.00. The lower initial pH is employed to gain greater retention of aspartate and glutamate from the void response, as well as early eluting neutral background peaks. Transition to 6.8 results in near optimum separation during the remainder of the run. Minor adjustments to this scheme may be required to compensate for small intercolumn variations or matrix specific impurities. In particular, desired placement of GABA between taurine and the valine/methionine doublet may be required. Between pH 6.50 and 6.80 GABA can be moved fairly selectively, toward valine at lower values and toward taurine at higher values. Over this window, however, most of the remaining separation will stay intact. Above 6.80 glycine and arginine may move together. Sometimes a value of 6.5 to 6.6 for the pH of the B and C buffer is better.

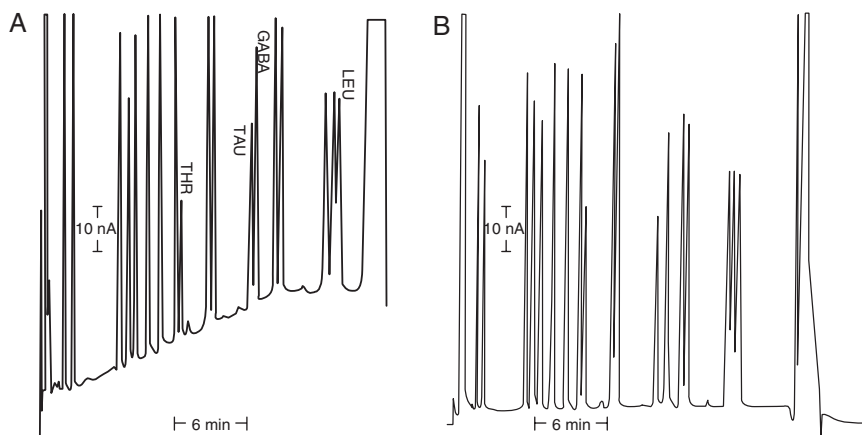
9.9 SPECIAL PROBLEMS

The separation is quite stable as outlined. If relatively minor changes in the recommended solvent program do not provide adequate separation, it may indicate a column problem. In such a case the column should be rewetted, equilibrated, and the separation attempted again. If problems still exist, contact BAS for technical assistance.

Baseline stability with this method is excellent but drift is not totally absent. The tetrahydrofuran is generally the primary contributor (Figure 9.3 A and B). "Bad" tetrahydrofuran will cause baseline drift that roughly parallels the proportioning of C phase into the system and may also accentuate baseline movement during the pH changeover. This effect tends to be cumulative, becoming progressively worse with each run. Electrode fouling is also common. Indicators of mobile phase problems are background currents greater than 10–15 nA at 100% A and baseline movement (from the start to just before stripping peak) of more than 1–3 nA. The use of BAS "EC-Certified" reagents is strongly encouraged in order to minimize down time due to these problems.

Electrode fouling can be reduced by vigorously wiping the electrode with a methanol-soaked lab tissue each morning.

Figure 9.3. Chromatograms illustrating impact of THF quality. (A) Reagent grade THF, preservative free. Note the 25 nA increase in the background current as %C increases, and the larger than usual final "flushing peak." Under these conditions, electrode fouling is also common, but easily removed with methanol and lab tissue. GABA resolution could be improved by lowering the pH of B and C to approximately 6.6-6.7. (B) Same as Figure 9.3(A), but EC Certified THF. The baseline is flat and the flushing peak greatly reduced.



THF problems can be completely eliminated if you don't need to separate glycine and threonine. Simply use acetonitrile in place of THF. GLY and THR will co-elute, but the remainder of the chromatogram will be similar.

Section 10. METHOD II – ASPARTATE/GLUTAMATE

10.1 DESCRIPTION

The aspartate/glutamate method utilizes a solvent program designed to allow rapid determination of only these two amino acids. It is based on isocratic elution for the derivatives of interest followed by a step to higher solvent for stripping of the more strongly retained materials. The time between injections is 6–7 minutes for manual injections and 10 minutes for automated injections.

10.2 SYSTEM MODIFICATIONS

The system, system preparation, reagents, and derivatization protocol are as described in Method I and earlier sections of this manual. Make sure that the pulse damper is removed and that the bypass tube (from pressure transducer to purge valve) is installed in its place.

10.3 MOBILE PHASE PREPARATION

Make up 2 L of 0.1 M sodium acetate buffer, pH 6.00, as follows:

Add 11.5 mL glacial acetic acid to 1900 mL H₂O and adjust to pH 6.00 ±0.02, first with 6 M NaOH solution, then 0.1 M NaOH. Use of dilute base for the final adjustments will prevent overshooting the desired pH. However, small errors can be corrected by using dilute acetic acid with no ill effects on method performance.

Transfer the solution to a 2.0 L graduate, dilute to the mark, mix well, and filter the buffer through a 0.2 µm nylon membrane.

Note: The pH is the critical variable. Calibrate your pH meter according to the manufacturer's instructions just prior to titrating the acetate buffer to pH 6.00.

Then, make up the A and B solutions. Use graduated cylinders and not volumetric flasks.

Mobile Phase A

- 900 mL of 0.1 M acetate buffer, pH 6.00
- 100 mL of HPLC/UV grade acetonitrile

Mobile Phase B

- 100 mL of 0.1 M acetate buffer, pH 6.00
- 900 mL of HPLC/UV grade acetonitrile

Mobile Phase C

- 1000 mL H₂O, acetonitrile, or mixture thereof.
- Do not leave the mobile phase reservoir empty.

Do not re-filter these solutions.

Place the mobile phases in their respective reservoirs and sparge for 5–10 minutes with inert gas. Close the exhaust valve and pressurize the reservoirs to 2–3 psi. Warm all bottles to 35 °C and set the oven to 40 °C. Run a 20 minute purge at 50% A, 50% B against a 3000 psi pressure limit to flush out residual acetonitrile/water. Equilibrate the column for start up. Connect the column outlet to the cell and assemble the reference and working electrodes.

10.4 BAS 200A METHOD FILE

PUMP FILE:

High Pressure Limit	4000 psi
Low Pressure Limit	100 psi
Flow Rate	1.0 mL/min
Synchronized Runs	Yes
Autoruns	No

GRADIENT PROGRAM

Time (min)	%A	%B	%C
0.0	91	9	0
1.5	91	9	0
1.6	30	70	0
3.5	91	9	0
6.0*	91	9	0

*When 6.0 min has elapsed, the system is reequilibrated and the next injection may occur. The BAS 200A will return to an EQUILIBRATING mode until the CMA/200 Microsampler's ANALYSIS TIME has elapsed and the next sample has been derivatized and loaded into the loop.

EC DETECTOR FILE

Number of Electrodes	1 Glassy Carbon, Upstream Position
----------------------	---------------------------------------

DETECTOR PROGRAM

Time	LCEC	POT	GAIN	FLT	SIGN	RCDR
0.0	1	+700	+50 nA	0.1	+	0
3.0	1	+700	+500 nA	0.1	+	0
6.0	1	+700	+50 nA	0.1	+	0

TEMPERATURE FILE

Oven	40°C
Cell	20°C
Bottles A & B	35°C

TIMED EVENTS FILE

Time	Event 1*	Event 2	Event 3	Event 4
Default	0	0	0	0
0.0	1	1	0	0
0.1	0	0	0	0
2.0	0	0	0	0

10.5 CMA/200 METHOD FILE

Use the parameters listed in “CMA/200 Derivatization Protocol,” section 5.2, page 9, with the exception that CMA/200 ANALYSIS TIME be changed to about 3 min. The BAS 200A will sit idle for approximately 4 min. after each 6 min. run time.

10.6 FIRST RUNS

Equilibrate the BAS 200A's file, and set up 5 “reagent blank” runs to cycle through the gradient. This is accomplished by:

- Loading 5 septum-capped vials (filled with 10 μ L of water)
FIRST SAMPLE: 1
NO. OF SAMPLES: 5
NO. OF INJECTIONS/SAMPLE: 1
- Ensuring that the BAS 200A control software says EQUILIBRATING.
- Pressing START on the CMA/200's controller.

During this time detector background will stabilize, ultimately reaching a value less than 10–15 nA at the $t=0$ conditions.

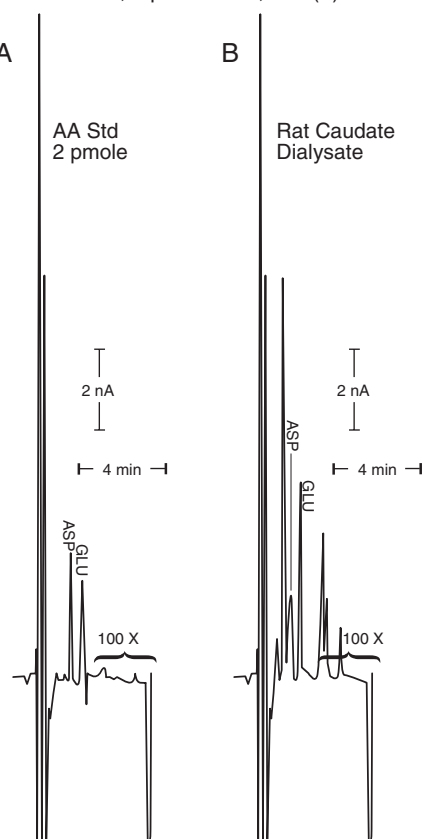
Now dilute the supplied 2.5 mM amino acid standard 400-fold with H₂O and use 10 μ L aliquots as samples 6–10. Repeat the above sequence.

The most stable and drift free performance is obtained for repetitive runs at equally spaced time intervals. Long intervals at low solvent strength may result in on-column concentration of impurities which will strip off the column during one or two subsequent runs. This stripping may result in poor baseline quality as well as discrete interfering peaks.

10.7 TYPICAL RESULTS

Typical chromatograms for this method are shown in Figure 10.1. The suggested gain change (to 500 nA between 3 and 6 minutes) is largely for the sake of appearance. It does prevent undesirable saturation of the detector output which can result in a lengthy electrode re-equilibration period.

Figure 10.1. Chromatograms of (A) Asp/Glu standard, 2 pmole each, and (B) rat caudate dialysate. Both run according to Method II.



10.8 SEPARATION ADJUSTMENT

The mobile phase pH can be adjusted to control resolution between aspartate and glutamate. Generally a pH between 6 and 6.5 gives the best selectivity for this derivative pair. At pH values much below 5.8–6.0, aspartate and glutamate begin to fuse and retain more strongly. Any alteration in mobile phase pH will likely require readjustment of the acetonitrile concentration in order to place the peaks within the desired elution window.

SECTION 11. METHOD III – GABA

11.1 DESCRIPTION

The third method is a non-gradient procedure optimized strictly for the determination of GABA. It utilizes the higher pK_a of the GABA carboxyl moiety relative to the other amino acids to selectively move GABA to a position behind the common amino acids. Run time with this method is approximate 12–15 minutes and is limited by the presence of several late eluting peaks. If only GABA is of interest, this may be the method of choice.

11.2 SYSTEM MODIFICATIONS

The system, system preparation, reagents, and derivatization protocol are as described in Sections 9, 10, and earlier sections of this manual.

Since isocratic (non-gradient) elution is used, extra connecting volumes can be inserted into the system without affecting elution reproducibility or sharpness. The 10 mL in-line pulse damper should be installed to ensure the lowest baseline noise.

11.3 MOBILE PHASE PREPARATION

Make up 1 L of 0.1 M sodium acetate buffer, pH 5.00, as follows:

Add 5.75 mL glacial acetic acid to 900 mL H₂O and adjust to pH 5.00 \pm 0.02, first with 6 M NaOH solution, then 0.1 M NaOH. Use of dilute base for the final adjustments will prevent overshooting the desired pH. Small errors can be corrected by using dilute acetic acid with no ill effects on method performance. Transfer the solution to a 1.0 L graduated cylinder, dilute to the mark, mix well, and filter the buffer through a 0.2 μ m nylon filter.

Then make up—using graduated cylinders—the A and B solutions:

Mobile Phase A

500 mL of 0.1 M sodium acetate buffer, pH 5.00
360 mL of HPLC/UV grade acetonitrile

Mobile Phase B

same as mobile phase A.

Mobile Phase C

1000 mL H₂O, acetonitrile, or mixture thereof.
Do not leave the reservoir empty.

Do not re-filter these solutions.

Place the mobile phases in their respective reservoirs and sparge for 5–10 minutes with inert gas. Close the exhaust valve and pressurize the reservoirs at 2–3 psi. Warm bottles A & B to 35 °C and set the oven to 20 °C. Run a 20 minute purge at 50% A, 50% B against

a 3000 psi pressure limit to flush out residual acetonitrile/water. Equilibrate the column for start up. Connect the column outlet to the cell.

11.4 BAS 200A METHOD FILE

PUMP FILE:

High Pressure Limit	4000 psi
Low Pressure Limit	100 psi
Flow Rate	1.0 mL/min
Synchronized Runs	Yes or No
Autoruns	No

GRADIENT PROGRAM

Time (min)	%A	%B	%C
0.0	50	50	0
13.0	50	50	0

EC DETECTOR FILE

Number of Electrodes	1 Glassy Carbon, Upstream Position
----------------------	---------------------------------------

DETECTOR PROGRAM

Time	LCEC	POT	GAIN	FLT	SIGN	RCDR
0.0	1	+700	+10 nA	0.1	+	0
13.0	1	+700	+10 nA	0.1	+	0

TEMPERATURE FILE

Oven	20°C
Cell	20°C
Bottles A & B	35°C

TIMED EVENTS FILE

Time	Event 1*	Event 2	Event 3	Event 4
Default	0	0	0	0
0.0	1	1	0	0
0.1	0	0	0	0
2.0	0	0	0	0

*When a CMA/200 Microsampler is used, this Event is reserved for BAS 200A↔CMA/200 communication.

11.5 CMA/200 METHOD FILE

Use the parameters listed in "CMA/200 Derivatization Protocol", section 5.2, page 9, with the exception that CMA/200 ANALYSIS TIME should be changed to about 7 min. If more time is needed for late-eluting peaks, lengthen this time, but leave the BAS 200A times unchanged.

11.6 FIRST RUNS

Equilibrate the BAS 200A's file, and wait for the background current to subside to less than 10 nA. Allow a few hours.

Set up 5 "reagent blank" runs. This is accomplished by:

- loading 5 septum-capped vials (filled with 10 μ L of water)
FIRST SAMPLE: 1
NUMBER OF SAMPLES: 5
INJECTIONS/SAMPLE: 1
- Ensuring that the BAS 200A control software says EQUILIBRATING.
- Pressing START on the CMA/200's controller.

Note the locations of peaks in these runs for future reference.

Now dilute the supplied 2.5 mM amino acid standard 400-fold with H₂O and use 10 μ L aliquots as samples 6–10. Change the FIRST SAMPLE parameter to 6 and repeat.

The mobile phase for this method should provide consistently low background currents (between 4–6 nA). Low background levels are essential for minimizing noise. Nearly all problems with high background can be traced to mobile phase contamination and this usually indicates a problem with one or more of the components used for mobile phase preparation. Less frequently, the column itself may contribute significant background. The latter can easily be checked by bypassing the column and comparing average background current with and without the column.

11.7 TYPICAL RESULTS

The chromatogram in Figure 11.1 shows a 10 pmole GABA peak in the presence of 100 pmole each mixed amino acids. GABA elutes at approximately 5 minutes under these conditions, behind even the most hydrophobic common amino acids. An artifact peak due to the reagent occurs at 13 minutes and this limits the effective throughput of the method. Figure 11.2 shows a blank run and a processed dialysate sample obtained from rat hippocampus.

Figure 11.1. Chromatogram of mixed amino acid standard. 10 pmole GABA, 100 pmole other amino acids.

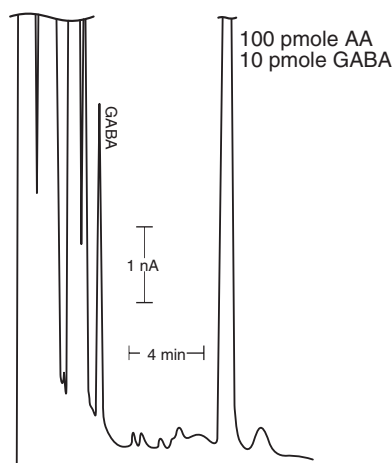
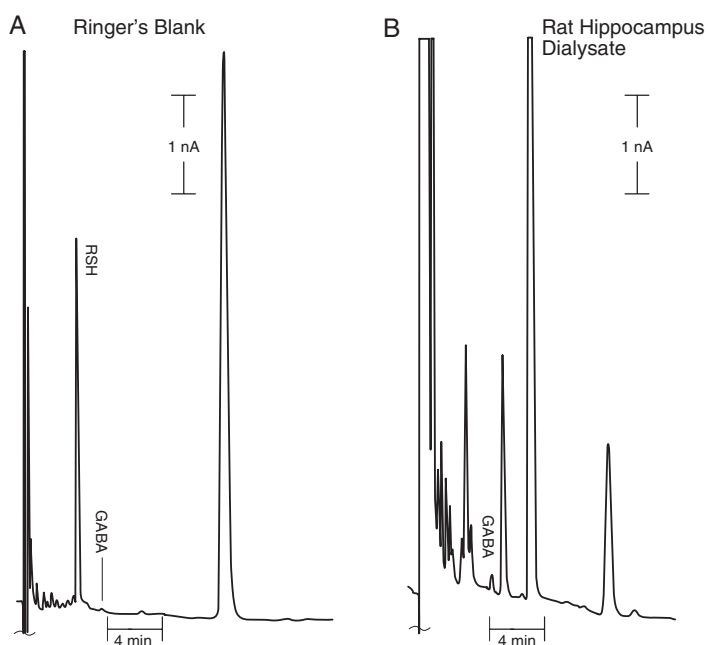


Figure 11.2. Chromatograms of (A) blank sample, (B) rat hippocampus dialysate. Both run according to Method III.



11.8 SEPARATION ADJUSTMENT

The amino acid peaks move dramatically as a function of pH under these conditions. GABA elutes behind the common amino acids only within a fairly narrow pH window: ~4.7–5.3. At pH 5.00 separation from the preceding group is nearly maximal. For this reason accurate and reproducible pH adjustment is essential. It may be that in certain samples the optimum pH will differ from that recommended. No more than 0.2 pH unit shifts should be made, however, in order to avoid excessive peak movement. As the pH is lowered from 5.0, all peaks will move outward, but GABA will move outward more slowly than others. Ultimately the hydrophobic amino acid derivatives will overtake it. GABA retention can also be adjusted via the acetonitrile concentration, but this affects all peaks in the same way.

11.9 SPECIAL PROBLEMS

Particularly when processing large numbers of dialysate samples, attention should be given to possible late eluting peaks that would cause problems with subsequent injections. In some matrices the run time may need to be lengthened. An alternative solution to this problem would be to employ the same strategy as outlined for in Section 10, i.e., the use of a high solvent stripping step at the end of the run.

Normally the latest eluting blank peak occurs at ~13.5 minutes, and this appears to be due to trace amounts of ammonia in the various solutions. Occasionally blanks will show a small peak at ~33 minutes, particularly with old reagent mixtures. While the identity of this peak is unknown, it is generally negligible with fresh reagents. Remember, however, that high sensitivity amino acid analysis is subject to many potential interferences due to the ubiquitous nature of amino acids and amines. Attention to cleanliness in all sample handling procedures is very important for trouble free operation.

Section 12. MICROBORE CHROMATOGRAPHY: THE EQUIPMENT

12.1 SETTING UP THE MICROBORE SYSTEM

The microbore system will be very similar to the standard system described on pages 6–7. The system is based on a BAS 200A Liquid Chromatograph, a CMA/200 Autosampler, and any of several systems for data collection and/or analysis. Please refer to pages 6–7 for instructions on setting up the major components of the system.

The microbore system differs from the standard system in the use of a SepStik microbore column (MF-8901), a flow-splitter kit (MF-8947), and a microbore injection valve (either our custom microbore injector MF-4161, or the CMA/200 Microsampler with a 5 or 10 microliter loop).

For systems with a manual injector, connect the SepStik and Flow Splitter as shown in Figure 12.1. Use Figure 12.2 as a guide for connecting the CMA/200 Microsampler to the microbore system. Use only narrow-bore tubing (0.005" ID) between the injector and the SepStik column, to minimize bandspread in the system.

Connect the SepStik column directly to the auxiliary electrode using a fingertight fitting, as shown in Figure 12.3. We recommend using a microbore filter frit (MF-8955) in the union at the top of the SepStik column. Microbore guard columns may be used if contaminants in samples become a problem. Kit MF-8943 includes six C₈ guard columns and a special nut for a no-dead-volume connection.

Figure 12.1. Connections incorporating the custom microbore injection valve.

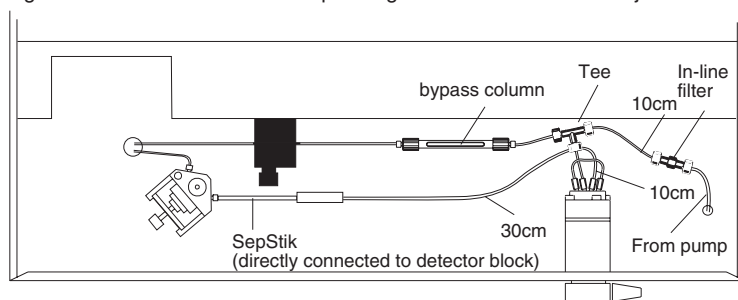


Figure 12.2. Connections incorporating the CMA-200 Microsampler.

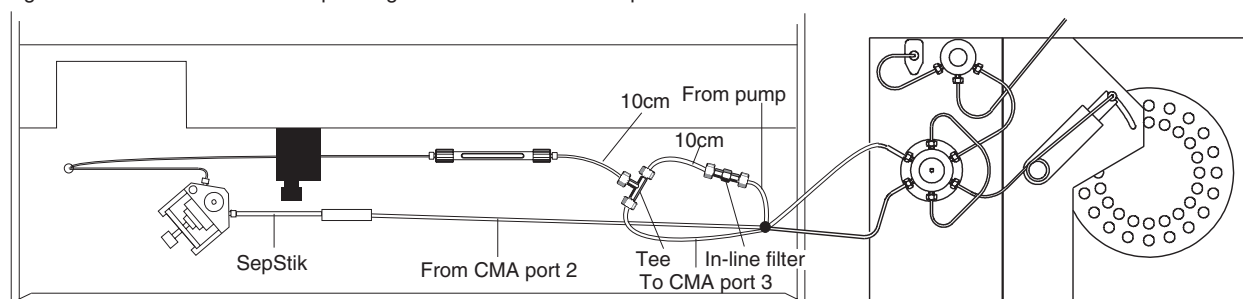
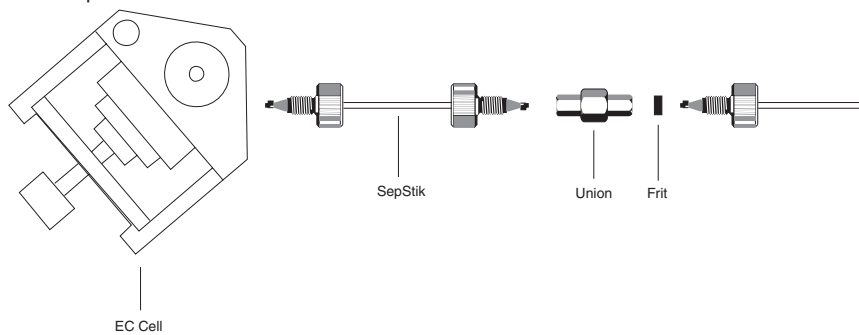


Figure 12.3. Detailed view of connection of SepStik column.



Be sure to use the special microbore gasket when attaching the glassy carbon working electrode. This gasket creates a flowcell with

Section 13. METHOD IV – PUTATIVE AMINO ACID NEUROTRANSMITTERS

13.1 DESCRIPTION

This microbore gradient-elution method allows for the simultaneous monitoring of all five major neurotransmitter amino acids: aspartate, glutamate, glycine, taurine and γ -aminobutyric acid (GABA). By default, it also allows for determination of asparagine, histidine, serine, arginine, threonine, tyrosine and alanine. Amino acids more hydrophobic than GABA are stripped during the column wash cycle. Total run time for the method (injection to injection) is about 40 minutes.

13.2 SYSTEM MODIFICATIONS

The system is configured as described previously (pages 6–7 and 11–12). Specific changes to these methods are: 1) A BAS SepStik microbore column and flow-splitter kit are used, 2) specially formulated microbore reagents are used, and 3) the mobile phases and gradient schedule that follow are used.

13.3 MOBILE PHASE PREPARATION

Make up one liter of 0.1 M sodium acetate buffer, pH 6.8, as follows:

Add 5.75 mL glacial acetic acid to about 900 mL LC-grade water and adjust to pH 6.8, first with 6 M sodium hydroxide, then with 0.1 M sodium hydroxide. (These solutions should be made up fresh each week using LC-grade water.) Dilute this buffer to one liter with LC-grade water, then filter through a 0.2 μ m membrane.

Make up the following mobile phases using graduated cylinders:

Table 13.1. Required mobile phases.

	Mobile Phase A	Mobile Phase B
0.1 M Sodium Acetate	690 mL	75 mL
Methanol*	240	225
Dimethyl Acetamide*	70	200

Do not re-filter the mobile phases after adding the organic components. Put them immediately into the solvent reservoirs on the BAS 200A, and sparge with inert gas for 10 minutes. Then close the exhaust valve to pressurize the system at 4 psi. The mobile phases will stay freshest at cool temperatures and anaerobic conditions. Thorough sparging with inert gas should remove enough dissolved gas to prevent outgassing in the flow stream. However, if outgassing becomes a problem the mobile phases can be heated to 35°C.

* We use Burdick and Jackson Brand™ HPLC-grade solvents.

**13.4 BAS 200A METHOD
FILE****PUMP FILE:**

High Pressure Limit	4000 psi
Low Pressure Limit	100 psi
Flow Rate	0.8 mL/min
Synchronized Runs	Yes
Autoruns	No

GRADIENT PROGRAM

Time (min)	%A	%B	%C
0.0	100	0	0
4.0	100	0	0
22.0	70	30	0
27.0	30	70	0
27.1	5	95	0
32.0	5	95	0
32.1	100	0	0
39.0	100	0	0

EC DETECTOR FILE

Number of Electrodes	2 Glassy Carbon, Series/W1–upstream
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DETECTOR PROGRAM

Time	LCEC	POT	GAIN	FLT	SIGN	RCDR
0.0	1	+700	+50 nA	0.1	-	0
	2	+700	+200 nA	0.1	+	0
39.0	1	+700	+50 nA	0.1	-	0
	2	+700	+200 nA	0.1	+	0

TEMPERATURE FILE

Oven	40°C
Cell	20°C
All Bottles	20°C

TIMED EVENTS FILE

Time	Event 1*	Event 2	Event 3	Event 4
Default	0	0	0	0
0.0	1	1	0	0
0.1	0	0	0	0

**13.5 CMA/200 METHOD
FILE**

Use the parameters listed in “CMA/200 Derivatization Protocol,” section 5.2, page 9. Be sure to use the microbore reagents (CF-2102) rather than the standard reagents.

Press FLUSH a few times to thoroughly purge the microsampler. There should be no air bubbles in the connecting tubing or the syringes. Use vacuum-filtered 10% methanol in water as the flushing fluid, and change it daily. Remember, if the sample loop is to be underfilled, this fluid will be injected: use only LC–grade methanol and water.

13.6 FIRST RUNS

If the BAS 200A is not already running, start the pump and temperature controls, and equilibrate the detectors. You should do at least three runs before injecting a sample. First run the gradient without injecting anything. This will clean the column and strip off any contaminants that accumulated during isocratic operation.

The second run each day should be a derivatized sample of water. There should be no peaks where any amino acids of interest will elute. See section 8, page 15 for information about obtaining clean blanks.

The third run should be a set of standards. If you are using two detectors at different gains, at least two concentrations of standards must be injected.

13.7 TYPICAL RESULTS

Figure 13.1 shows a separation of amino-acid standards. The elution order shown should not be relied on absolutely, since slight changes in solvent composition, pH, or column packing could reverse the positions of closely eluting pairs. You should confirm the elution time of compounds of interest by injecting individual standards.

Figures 13.2-13.4 show separations of samples obtained by *in vivo* microdialysis.

Figure 13.1. Amino acid standards (5 microliter injection, 2 micromolar standards).

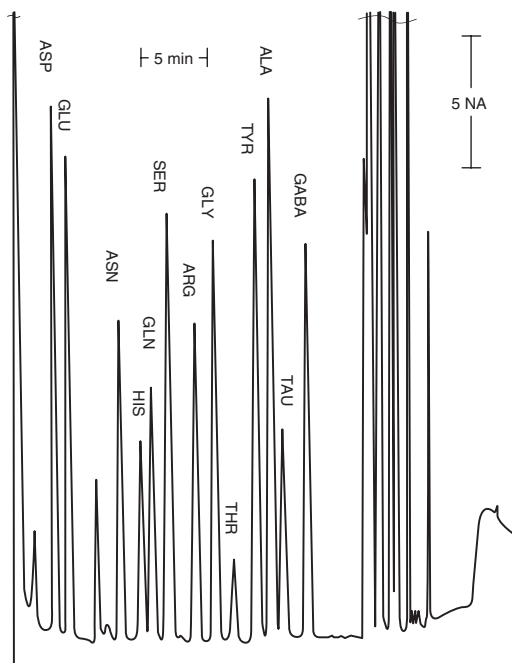


Figure 13.2. Microdialysis sample from rat brain (7 microliter injection).

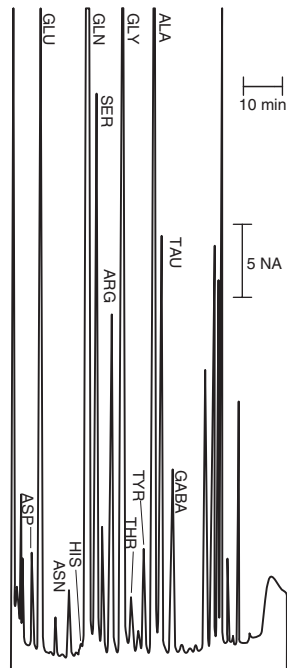


Figure 13.3. Microdialysis sample from rat jugular (1 microliter injection).

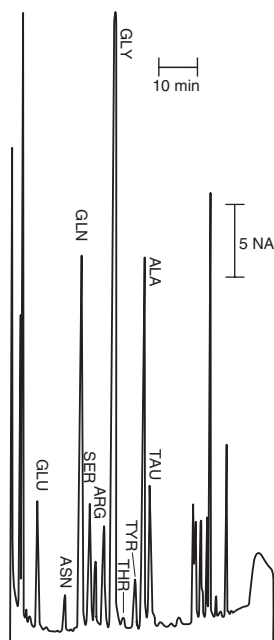
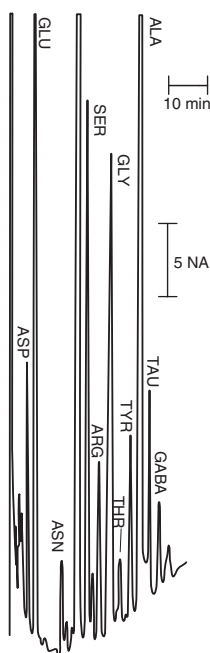


Figure 13.4. Microdialysis sample from dog brain (10 microliter injection).



13.8 SPECIAL PROBLEMS

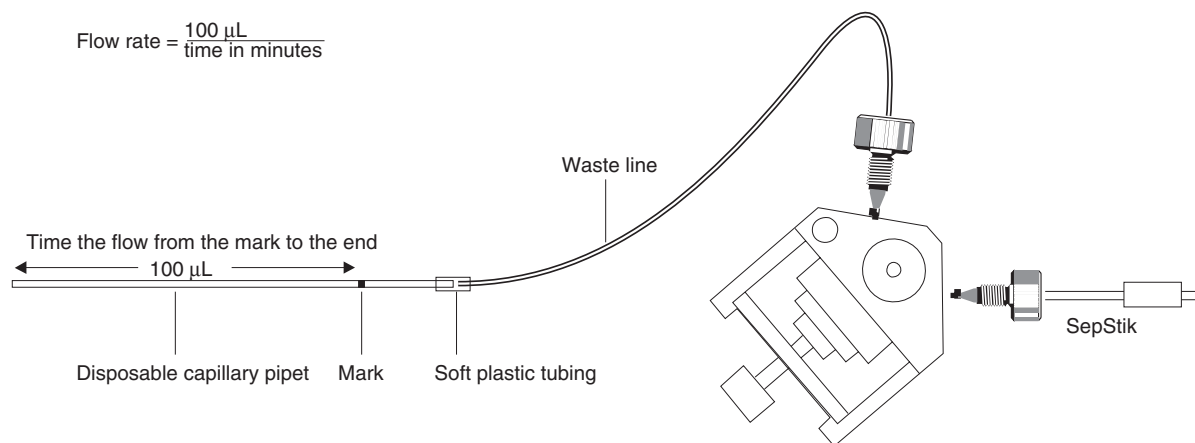
Baseline stability with this method is good, but some drift occurs, particularly as the mobile phase ages. Background currents should start out in the 10–15 nA range, but may drift into the 15–20 nA range after a few days. Make fresh mobile phase every few days, and clean the column by running the gradient.

Contamination from the mobile phase or the samples also may coat the working electrodes, causing high backgrounds and low response. The glassy carbon surfaces can be briskly wiped with a methanol-soaked lab tissue to remove any coating (turn them OFF first!). Re-equilibration takes about 30 minutes, and can occur in conjunction with a gradient run to clean the system each morning.

Flow rate through the SepStik column should be about 175 microliters per minute at a system flow rate of 0.8 mL per minute. (This rate will vary from column to column.)

Clogging in the microbore flow path can drastically affect the separation. If you suspect clogging, measure the flow rate as show in Figure 13.5.

Figure 13.5. Measuring flow rate.



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