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Requirements

for Acetylcholine analysis

using the ALEXYS® Neurotransmitters system (180.0091W)

180.7064u, Edition 5, 2022

Warning Symbol

The warning sign denotes a warning. It calls attention to a procedure or practice which, if not adhered to, could result in costs, damage or destruction of parts or all of the equipment. Do not proceed beyond a warning sign until the indicated conditions are fully understood and met.

For research purposes only. **The ALEXYS system is not tested by the manufacturer to comply with the In Vitro Diagnostics Directive.**

Observe safety

Operation of an electrochemical detector can involve the use of hazardous materials including corrosive fluids and flammable liquids. The instrument should only be operated by users with the following expertise:

- Completed degree as chemical laboratory technician or comparable vocational training
- Fundamental knowledge of liquid chromatography
- Knowledge and experience in the safe handling of toxic and corrosive chemicals and knowledge of the application safety measures prescribed for laboratories.
- Participation in an end-user training (daily use of system and chromatography software) performed by the manufacturer or a company authorized by the manufacturer.

Unskilled, improper, or careless use of the instrument and the related chemicals can create fire hazards, or other hazards which can cause death, serious injury to personnel, or severe damage to equipment and property.

Observe all relevant safety practices at all times.

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C H A P T E R 1

Introduction

Thank you for ordering an ALEXYS LC-ECD system. For a successful on-site installation of the ACh analysis on the ALEXYS system, please arrange the following requirements at your location in advance:

- a computer (see document 195.7000 for the PC requirements)
- general laboratory conditions and facilities, consumables and chemicals for use with the ALEXYS system (see document 180.7070C)
- application specific chemicals and consumables listed in this document

Arrange these requirements well in advance before the installation to prevent (costly) delays.

This document lists the application specific chemicals for the analysis of ACh using the combination of the following hardware:

ALEXYS® Neurotransmitters system (180.0091U)

ALEXYS ACh kit (p/n 180.0505)

AChE/ChOx IMER, 1mm (pn. 250.3532)

The IMER contains enzymes and needs to be stored at 4°C immediately after receipt.

Have the chemicals and solutions ready at the start of the installation.

C H A P T E R 2

Chemicals

For LC-ECD applications, only chemicals of sufficient specific quality should be used to be able to have an optimal system with good performance. The appendix shows detailed descriptions of some of the chemicals that have been used in the Antec R&D laboratory, as an example of what works.

15% HNO_3 for passivation/sterilisation

Chemicals

- 65% HNO_3 in water (commercial solution)
- Water (Resistivity >18MOhm.cm, TOC<10ppb)

Preparation

- 60 mL water + 20 mL 65% HNO_3

10% acetonitrile for column flushing

Chemicals

- Acetonitrile
- Water (Resistivity >18MOhm.cm, TOC<10ppb)

Preparation

1. 10 mL acetonitrile diluted with water up to total volume of100 mL
2. degas in sonic bath for 15 min

Standards

Chemicals

- Standards of Acetylcholine and Choline in high purity grade
- Water (Resistivity >18MOhm.cm, TOC<10ppb)

Preparation

- 10 mL 1 mM of each individual standard in water

Store at 4 °C until use (max 1 month)

Calibrator solutions

Chemicals

- □ Chemicals for preparation of perfusion fluid
- □ Water (Resistivity >18MOhm.cm, TOC<10ppb)

Preparations

- □ 100 mL microdialysis perfusion fluid
  Store at 4 °C until use (max 1 month)

Suggestion for perfusion fluid composition:

Component	Concentration (mM)
NaCl	147
KCl	3
$MgCl_2.6H_2O$	1.2
$CaCl_2$	1.2

- □ 0, 2, 4, 6, 8 and 10 nM ACh in a background of water
- □ 0, 2, 4, 6, 8 and 10 nM ACh in a background of perfusion fluid.
  Store at 4 °C until use (max 1 day)

Mobile phase

For separation of acetylcholine with column Acquity UPLC HSS T3 50 x 1 mm column, 1.8 µm (pn. 250.1160):

Mobile phase composition	50 mmol/L sodium dihydrogen phosphate 0.5 mmol/L EDTA.Na_2 pH 7.5 set with 50 %NaOH 1.6 g/L octanesulfonic acid, sodium salt 0.5 mM tetramethylammonium chloride

Note: this mobile phase is a suggested starting condition and not guaranteed to give best results for all kinds of unknown samples. Condition optimisation may be necessary.

Chemicals

- □ Water (Resistivity >18MOhm.cm, TOC<10ppb)
- □ Di sodium ethylenediaminetetraacetic acid (EDTA. Na_2)
- □ Sodium dihydrogen phosphate
- □ 50% w/w NaOH in water (commercial solution)
- □ Octane sulphonic acid, sodium salt (OSA)
- □ Tetramethylammoniumchloride

Preparation

1. Add about 0.9 L de-ionized low-TOC water in an empty and clean glass beaker and add a clean stir bar
2. Add 0.1861 g $Na_2EDTA.2\ H_2O$ and stir till it is completely dissolved

3. Add 6.00 g NaH_2PO_4 and stir till it is completely dissolved.
4. Set the pH of the solution to 7.5 using 50% NaOH solution.
5. Add de-ionized low-TOC water to a total volume of 1.0 L
6. Add 1.6 g octanesulfonic acid sodium salt
7. Add 55 mg tetramethylammoniumchloride
8. Degas the mobile phase in a sonic bath for about 15 minutes.

The mobile phase can be stored in the fridge at 4 °C for a week.

Do not filter the mobile phase; the 0.2 µm inline Whatman filters present in the low pressure solvent lines of the ALEXYS system will take care of filtering.

A small batch of the mobile phase can be poured in a smaller bottle and connected to the system; visually inspect the mobile phase daily for microbial growth. Refresh mobile phase that is standing in room temperature **at least every 3 days** or more often if microbial growth is observed.

0.1 M phosphate buffer, pH 8.2 for IMER storage

Chemicals

- Water (Resistivity >18MOhm.cm, TOC<10ppb)
- Sodium dihydrogen phosphate
- 50% w/w NaOH in water (commercial solution)

Preparation

1. Add about 90 mL L de-ionized low-TOC water in an empty and clean glass beaker and add a clean stir bar
2. Add 1.20 g NaH_2PO_4 and stir till it is completely dissolved.
3. Set the pH of the solution to 8.2 using 50% NaOH solution.
4. Add de-ionized low-TOC water to a total volume of 100 mL
5. Degas the solution in a sonic bath for about 15 minutes.

This solution can be stored in the fridge at 4 °C for a month.

Flush the IMER with this solution before storing the IMER in the fridge.

CHAPTER 3

Procedures

System start-up for new hardware parts

Organic solvents will damage the IMER, and therefore water has to be used for needle wash and piston wash.

Flush out pump, pulsedamper, tubing and injector

During the first 4 steps, flush out the system from the pump up to the injector valve, both in INJ and LOAD position
Do not run the acid through the in-line filter nor degasser!

1. 50 mL H_2O
2. 50 mL 15% HNO_3 in water
3. 50-100 mL H_2O, until pH>3
4. 50 mL 10% acetonitrile

Flushing a new column

5. Connect the degasser, in-line filter and column
6. Flush 10 bed volumes of 10% acetonitrile through the column
7. Run mobile phase through the column for about 16 h @ 50 µL/min

Connect the IMER

8. Run mobile phase through the IMER for about 10 min @ 50 µL/min

Connect detector

9. Prefill the flow cell with mobile phase and connect to the IMER
10. Run mobile phase through until no bubbles come out of the flow cell
11. Attach waste line

System start-up for already used parts

Organic solvents will damage the IMER, and therefore water has to be used for needle wash and piston wash.

Flush out whole system up to injector

1. 50 mL 10% acetonitrile

Connect the column

2. Flush 10 bed volumes of 10% acetonitrile through the column
3. Flush at least 50 bedvolumes mobile phase through the column

Make sure at this point to have water as needle wash and piston wash.

Connect the IMER

4. Run mobile phase through the IMER for about 10 min @ 50 µL/min

Connect detector

5. Prefill the flow cell with mobile phase and connect to the IMER

6. Run mobile phase through until no bubbles come out of the flow cell
7. Attach waste line

Signal activation procedure

When the cell is new or had been stored some time apply the following before measurements:

Activation pulse

- Setting: PAD mode
- Duration:10 min
- E1=+1.0V, E2=-1.0V, t1=1000ms, t2=1000ms, ts=20ms

Stabilization

- Setting: DC mode
- Duration:30-60 min
- Ecell =+0.2V
- Icell should drop below 25 nA in less than 20 min.

Signal reactivation procedure

Do not polish the Sencell Pt working electrode surface.
When the S/N ratio has dropped below a workable result, apply the following reactivation pulse and recalibrate the signal:

Reactivation pulse, programmable as timed event

- Setting: DC mode
- E=-0.5V for 0.2 min followed by E=0.8V for 0.2 min
- Signal stabilization time at working potential: at least 10 min

System stand-by

If the system will be used every day or every few days, leave the flow cell on, and recycle the mobile phase between measurements. However, refresh mobile phase in the bottle every week.

System shut down

1. Disconnect the flow cell and store dry after cleaning
2. If you plan to use the IMER again in less than a month, you make store the IMER in mobile phase at 4 °C.
3. If the IMER will not be used within a month, flush it with 0.1M phosphate buffer (pH 8.2) before storing at 4 °C.
4. Flush the analytical column with 10 bed-volumes 10% acetonitril and followed by 10 bed-volumes 50% acetonitrile.
5. Cap and store the column
6. Replace the solutions of the needle wash, piston wash and mobile phase bottle with 20% Methanol. Flush the instruments and shut down.

A P P E N D I X

A list of the application specific chemicals with purity and purchase details is shown below as a guideline. The listed brands/purities are not necessarily the best chemicals, but these have been giving good results at the Antec R&D laboratory.

If for any reason alternative chemicals need to be purchased, be aware that chemicals that have a specification of high purity may have been tested for UV-active impurities, which can mean that they may still contain electrochemically active impurities. This is one of the reasons why general 'HPLC grade' water is not suitable for use with EC detection:

- choose chemicals with the same purity or better
- do not choose ultra dry grade chemicals

Table 1. Brands and purities of chemicals used for application development at Antec.

Component	Purity	Brand	Order no:	Mw	kg/L
Sodium dihydrogen phosphate	BioXtra,≥99.0%	Sigma-Aldrich	S2828	119.98	
1-Octane sulphonic acid, sodium salt (OSA)	HPLC grade	Acros	384771000	216.28	
Tetramethylammonium chloride	puriss., p.a., for ion pairing chromatography	Fluka	74202	109.6	
NaOH, 50% w/v in water	puriss., p.a., for HPLC	Fluka	71686	40.00	D:1.54
$Na_2EDTA.2H_2O$	SigmaUltra, 99%	Acros	147855000	372.23	
HNO_3	65% solution	Fluka	84380	63.01	D:1.40
Acetonitrile	HPLC grade, 99.9%	Acros	268260025	41.05	D:0.781
Methanol	HPLC gradient grade	Baker	8402	32.04	D:0.79
Water	TOC <10ppb and deionised, resistivity >18 MOhm-cm (Barnstead Easypure II)				

Manufacturers

JT-Baker	http://www.avantormaterials.com
Sigma-Aldrich	http://www.sigmaaldrich.com
Fluka	http://www.sigmaaldrich.com
Fisher Scientific	http://www.fishersci.com
Barnstead	http://www.thermoscientific.com