

ADRA Kit

HPLC conditions and data analysis method

1. HPLC Analysis

1. Install the appropriate column in the HPLC system, prime and equilibrate the entire system with the Mobile Phase A and Mobile Phase B at column temperature of 40°C. The HPLC analysis is performed using a flow of 0.3 mL/min. and a linear gradient from 30% to 55% acetonitrile for NAC and from 25% to 45% acetonitrile for NAL within 10 minutes, followed by a rapid increase to 100% acetonitrile to remove other materials. Refer “1-1. HPLC Conditions” for details on the gradient.

Note: The 6.5 minutes re-equilibration time was determined using a Shimadzu Prominence HPLC system. Other systems may require more or less re-equilibration time due to system mixing quantity. Shorter equilibration times will be acceptable if peak retention times are stable.

1-1. HPLC Conditions

Column	FUJIFILM Wako Pure Chemical Corporation Wakopak® Core C18 ADRA ϕ 3.0 × 150 mm [Cat. # 233-63991]
Mobile phase	A: 0.1%(v/v) TFA in water B: 0.1%(v/v) TFA in acetonitrile
Column Temperature	40°C
Test chemical solution Temperature	25°C If the auto-sampler has a cooling function, test chemical solutions can be kept more stable at 4°C.
UV detector*1	Photodiode array detector (for example, Shimadzu SPD-M20A) or absorbance detector (281 nm)
Injection Quantity	10-20 μ L (The injection quantity varies according to HPLC system. If peaks are too broad, the injection quantity should be decreased.)
Run Time	20 minutes

Flow Conditions	NAC flow conditions			
	Time	Flow	%A	%B
	0 min.	0.3 mL/min.	70	30
	9.5 min.	0.3 mL/min.	45	55
	10 min.	0.3 mL/min.	0	100
	13 min.	0.3 mL/min.	0	100
	13.5 min.	0.3 mL/min.	70	30
	20 min.	End run		
	NAL flow conditions			
	Time	Flow	%A	%B
	0 min.	0.3 mL/min.	75	25
	9.5 min.	0.3 mL/min.	55	45
	10 min.	0.3 mL/min.	10	100
	13 min.	0.3 mL/min.	10	100
	13.5 min.	0.3 mL/min.	75	25
	20 min.	End run		

Note 1: The mixer quantity should be verified and adjusted in advance because the appropriate elution pattern of NAC/NAL peak will not be shown if the mixer quantity for mixing each mobile phase is not appropriate (For example, 0.5 mL mixing quantity is appropriate for Shimadzu prominence HPLC system).

Note 2: The inner diameter of pipe and the length of pipe from column outlet to detector inlet must be less than 0.18 mm and less than 50 cm, respectively, because the peak of NAC/NAL might be broadened depending on inner diameter and length of pipe.

Note 3: If more than one wavelength is detected, also 291 nm besides 281 nm should be detected to check out peak purities of NAC/NAL.

1-2. HPLC Sample Analysis Sequences

Each sample of HPLC analysis should be analyzed in number order below. Refer to the table showing Examples of HPLC Sample Analysis Sequences for more practical sequences about HPLC analysis.

1. Start to analyze calibration standards and Reference Control A (n = 3).
2. The Co-elution Control does not need to be analyzed by turns if it is analyzed after analysis of standard solution and Reference Control A.
3. Reference Control B should be analyzed three times (total six times) before and after the analysis of sample, Reference Control C and Positive Control.
4. The Reference Control C, Positive Control and Test chemical solutions are analyzed. (After the first set of replicates of each sample is analyzed, the second set of replicates of each should be analyzed)

Example of HPLC Samples Analysis Sequences

(A more specific analysis sequence can be found at the last page.)

Std 7 (Buffer solution for dilution) [†] Std 6 Std 5 Std 4 Std 3 Std 2 Std 1	Ref. 4-1. 1)
Reference Control A, n=1 Reference Control A, n=2 Reference Control A, n=3	Ref. 4-1. 1)
Co-elution Control 1 Co-elution Control 2 Co-elution Control 3 ... Co-elution Control n	Ref. 4-4.
Reference Control B, n=1 Reference Control B, n=2 Reference Control B, n=3	Ref. 4-2.
Reference Control C, n=1 ^{§†} Phenylacetaldehyde/Squaric Acid Diethyl Ester (Positive Control), n=1 Test chemical solution 1, n=1 Test chemical solution 2, n=1 Test chemical solution 3, n=1 ... Test chemical solution n, n	<i>First set of replicates</i> Ref. 4-1. 2), 4-1. 3), 4-2.
Reference Control C, n=2 ^{§†} Phenylacetaldehyde/Squaric Acid Diethyl Ester (Positive Control), n=2 Test chemical solution 1, n=2 Test chemical solution 2, n=2 Test chemical solution 3, n=2 ... Test chemical solution n, n=2	<i>Second set of replicates</i> Ref. 4-1. 2), 4-1. 3), 4-2.
Reference Control C, n=3 ^{§†} Phenylacetaldehyde/Squaric Acid Diethyl Ester (Positive Control), n=3 Test chemical solution 1, n=3 Test chemical solution 2, n=3 Test chemical solution 3, n=3 ... Test chemical solution n, n=3	<i>Third set of replicates</i> Ref. 4-1. 2), 4-1. 3), 4-2.

Reference Control B, n=4	Ref. 4-2.
Reference Control B, n=5	
Reference Control B, n=6	

† Start to analyze calibration standard immediately after addition of Reaction Fixing Solution and preparation of dilution series of standard solution.

§ Analyze three replicates for Reference Controls C. These results are used to calculate the NAC/NAL depletion in each solvent and to verify that solvent used does not affect NAC/NAL depletion.

2. DATA ANALYSIS & CALCULATIONS

The concentration of NAC/NAL is calculated from peak area of absorbance at 281 nm for each test chemical solution based on the calibration curve derived from standard solutions Std 1 to Std 7. NAC/NAL percent depletion is calculated by dividing NAC/NAL peak area of each test chemical solution by mean peak area of Reference Control C.

2-1. Calculation of Peak Area of NAC/NAL

Integrate the appropriate peaks and determine peak area for standards, test chemical solution and controls. The peak area of each integrated peak must be reported.

2-2. Calculation of Concentration of NAC/NAL

2-2-1. Generate a linear calibration curve based on the concentration of standards and the peak area. Suitable calibration curves must have an $r^2 > 0.990$.

2-2-2. Calibrate the mean NAC/NAL concentrations in Reference Controls A and C, SD and CV. Mean of each concentration should be 3.2–4.4 μM . The NAC/NAL concentration of Reference Controls A and C must be reported.

2-2-3. Calculate the mean NAC/NAL peak area, SD and CV for the Reference Controls C (n=3) for each solvent used. Mean of each concentration should be 3.2–4.4 μM . However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM .

2-3. Calculation of Peak Area of NAC/NAL

2-3-1. Calculate the mean NAC/NAL peak area for the six Reference Controls B and the three Reference Control C in acetonitrile, SD and CV. The CV must be less than 10%.

2-3-2. Calculate the mean NAC/NAL peak area at 281nm for the three Reference Controls C.

2-4. Calculation of Percent Depletion of NAC/NAL

2-4-1. For the Positive Control and for each test chemical, calculate the Percent NAC/NAL Depletion in each replicate from the NAC/NAL peak area of the replicate injection and the mean NAC/NAL area in the three relevant Reference Controls C (in the appropriate solvent), using the following formula.

Percent NAC/NAL Depletion (% depletion) = [1- (NAC/NAL Peak Area in Replicate Injection/mean NAC/NAL Peak Area in Reference Controls C)]×100

2-4-2. The mean Percent NAC/NAL Depletion (Average score) of the three replicate determinations, SD and CV should also be calculated and reported. Report results to one decimal place.

3. DATA REPORTING (FOR NAC AND NAL)

System Suitability

- NAC/NAL peak area at 281 nm of Standard and Reference Control B and C replicate.
- The linear calibration curve should be graphically represented and the r^2 reported.
- NAC/NAL concentration (μM) of Reference Control A replicate.
- Mean NAC/NAL concentration (μM) of Reference Controls C replicate, SD and CV.

Reference Controls:

- NAC/NAL peak area at 281 nm of Reference Control B and C replicate.
- Mean NAC/NAL peak area at 281 nm of the nine Reference Controls B (n = 6) and C (n = 3) in acetonitrile, SD and CV (for stability of Reference Controls over analysis time).
- For each solvent used, the mean NAC/NAL peak area at 281 nm of the three appropriate Reference Controls C replicate (for calculation of Percent NAC/NAL Depletion).
- For each solvent used in this assay, the mean NAC/NAL concentration (μM) of the appropriate Reference Control C replicate, SD and CV.

Positive Control (Phenylacetaldehyde)

- NAC/NAL peak area at 281 nm of each replicate.
- Percent NAC/NAL Depletion of each replicate.
- Mean NAC/NAL Depletion of the three replicates, SD and CV.

For Each Test Chemical:

- Solvent chosen
- Appearance of precipitate in the reaction mixture at the end of the incubation time.
It must be reported if precipitate was re-solubilized or centrifuged.
- NAC/NAL peak area at 281 nm of each replicate (for systems equipped with a PDA detector the peak area at 291 nm should also be reported).
- Percent NAC/NAL Depletion of each replicate.
- Mean of Percent NAC/NAL Depletion of the three replicates, SD and CV.

4. ACCEPTANCE CRITERIA

4-1. Acceptance Criteria for Amino acid Derivative Reactivity Assay Run

All criteria must be met for the whole run to be considered valid. If three criteria are not met, the run must be repeated for all test chemicals.

1) System Suitability:

Calibration Linearity $r^2 > 0.990$

Mean NAC/NAL concentration of Reference Controls A = 3.2–4.4 μM

2) Positive Control:

The mean Percent NAC/NAL Depletion value of the three replicates for Phenylacetaldehyde or Squaric Acid Diethyl Ester must fall within the range reported in the following table (Based on mean ± 3 SD from background data):

Positive Control	Percent NAC Depletion		Percent NAL Depletion	
	Lower Bound	Upper Bound	Lower Bound	Upper Bound
Phenylacetaldehyde or Squaric Acid Diethyl Ester	30	80	70	100

Maximum Standard Derivatives for Positive Control replicate:

Standard Deviation for Percent NAC Depletion must be $< 10\%$

Standard Deviation for Percent NAL Depletion must be $< 10\%$

3) Stability of Reference Controls over analysis time:

For each solvent used, the mean of the NAC/NAL concentrations of the three appropriate Reference Controls C = 3.2–4.4 μM . However, if 5% DMSO/Acetonitrile is selected as a solvent for test chemical, the mean of concentration of NAC should be 2.8–4.0 μM , as it is known that concentration of NAC decreases because of oxidation of SH group by DMSO.

4-2. Acceptance Criteria for Each Test Chemical

All criteria must be met for the run to be considered valid for a particular test chemical. If these criteria are not met, the run must be repeated for the test chemical.

1) Maximum Standard Deviation of Test Chemical Solution Replicates:

Standard Deviation for Percent NAC Depletion must be $< 10\%$

Standard Deviation for Percent NAL Depletion must be $< 10\%$

2) Reference Controls B and C in acetonitrile in the Analysis Sequence:

CV of NAC/NAL peak areas for the nine Reference Controls B ($n = 6$) and C ($n = 3$) in acetonitrile must be $< 10\%$.

3) Reference Controls C in the Analysis Sequence:

CV of NAC/NAL peak areas for the Reference Controls C ($n = 3$) in each solvent must be $< 10\%$.

4-3. Data Acceptance for Amino Acid Derivative Reactivity Assay

The average score should be calculated from depletions of NAC/NAL, and the test chemicals should be predicted to be either a Sensitizer or a Non-sensitizer according to following table.

NAC/NAL Prediction Model

Average score	Judgement
Less than 4.9%	Non-sensitizer
4.9% or higher	Sensitizer

If an average score for NAC depletion in a test chemical falls within the borderline range described below, additional testing should be performed to confirm the validity of the prediction. If the result of the second test is not concordant with the first test, a third test should be performed to determine a prediction for the test chemical by majority of the three test results.

NAC and NAL prediction model: $3.0\% \leq \text{average score} \leq 10.0\%$

NAC only prediction model: $4.0\% \leq \text{NAC depletion} \leq 11.0\%$

4-4. Handling of Co-elution

4-4-1. Co-elution: Interference

- (1) Some test chemicals will co-elute with the NAC or NAL. In order to detect possible co-elution of the test chemicals with NAC or NAL, the test chemicals included in the run must be injected alone (“Co-elution Controls”) at the beginning of the run sequence and their chromatograms compared to the chromatograms of Reference Controls C in the appropriate solvent.
- (2) If a chemical absorbs at 281 nm and has a similar retention time as a peptide (overlap of valley-to-valley integration periods), then verify whether or not the peak of test chemical is actually separated from the peak of NAC or NAL. If the peak of test chemical is completely overlapped with the peak of NAC or NAL, and if the boundary of two peaks (valley between peaks) is located higher than baseline, co-elution of the test chemical with that NAC or NAL should be reported. The “interfering” chemical peak should have a peak area that is $>10\%$ of the mean NAC/NAL peak area in the appropriate Reference Control. If co-elution occurs and proper integration and calculation of NAC/NAL depletion is not possible, the data should be recorded as “interference” for NAC/NAL the chemical co-elutes with.

Even if the test chemical does not co-elute with NAC or NAL, the Percent NAC/NAL Depletion can appear to be $< -10\%$ if the concentration of Reference Control C is comparatively low. Moreover, the Percent NAC/NAL Depletion can also appear to be $< -10\%$ due to inappropriate handling of the measurement. In such cases, retesting of

the test chemicals in question or other appropriate measure should be taken.

4-4-2. Peak purity of NAC/NAL: Area ratio of 281/291 nm

When a Photodiode Array detector is used, co-elution of chemical and NAC/NAL may also be verified by looking at the UV spectrum at 291 nm addition to 281 nm and calculating the area ratio of 281/291. This value should be consistent over all test chemical solutions and standards for a distilled NAC/NAL peak and thus gives a measure of peak purity. For each test chemical solution, a ratio in the following range would give a good indication that co-elution has not occurred. However, calculation of peak purity (area ratio of 281/291) might not always be possible, particularly if the test chemical is highly reactive with the NAC/NAL leading to very small peaks.

90% < Mean Area ratio of Reference Control < 110%.

4-4-3. Co-elution: Depletion < -10%

- (1) If the Percent NAC/NAL Depletion is < -10%, it should be considered that this may be a situation of co-elution, inaccurate NAC/NAL addition to the reaction mixture or just baseline noise. If the NAC/NAL peak appears at the proper retention time and has the appropriate peak shape, the peak can be integrated. In this case, there may just be baseline noise causing the NAC/NAL peak to be bigger or there may be some co-elution/overlap in retention time of the NAC/NAL and test chemical.
- (2) The calculated %-depletion should be reported as an estimate. In cases where a test chemical co-elutes with NAL, the NAC only prediction model can be used. In cases where a test chemical co-elutes with both NAC/NAL, the data should be reported as an inconclusive.
- (3) In cases where the test chemical co-elutes with the NAC and the peak of NAC cannot be integrated, the skin sensitization of test chemical cannot be predicted from the NAL depletion alone, and the data should be reported as inconclusive.

4-4-4. Calculation of peak area for co-elution

- (1) If the peak of NAC/NAL and the peak of test chemical partially overlap, the peak area of NAC/NAL should be integrated from valley of both peaks to baseline vertically.
- (2) If the peak of NAC/NAL and the peak of test chemical completely overlap, the data should be reported as an Inconclusive, and the peak area should not be calculated.

4-4-5. Estimated depletion values

In some cases, a test chemical might co-elute with NAC and/or NAL though the test chemical react with NAC and/or NAL. If this is the case, co-elution will make the peak area of NAC/NAL appear to be larger than it really is, therefore the calculated percent depletion may be lower than the true value. When the overlap in retention time between the test chemical and NAC/NAL is incomplete, percent depletion can still be calculated with a notation of “co-elution – percent depletion estimates”. If the average score is below

the criteria, the result should be reported as Inconclusive. However, unless NAC co-elutes with test chemical, the NAC-only prediction model should be used.

Average score	No co-elution	Co-elution with NAC alone or NAC and NAL	Co-elution with NAL only
< 4.9%	Non-sensitizer	Inconclusive	Apply NAC-only prediction model
4.9% \leq	Sensitizer	Sensitizer	Apply NAC-only prediction model

NAC Only Prediction Model

NAC Depletion	Judgement
less than 5.6%	Non-sensitizer
5.6% or higher	Sensitizer

Example: HPLC Analysis

There are 5 test chemicals. Chemical 1,2 and 3 are soluble in acetonitrile. Chemical 4 and 5 are soluble in distilled water.

The following 96-well Microwell Plate should be set up:

Std 7 (Dilution buffer blank)

Std 6

Std5

Std5

Std4

Std 3

Std 2

Std1

Reference Control A, n= 1 (made with acetonitrile)

Reference Control A, n= 2 (made with acetonitrile)

Reference Control A, n= 3 (made with acetonitrile)

Co-elution Control for Chemical 1

Co-elution Control for Chemical 2

Co-elution Control for Chemical 3

Co-elution Control for Chemical 4

Co-elution Control for Chemical 5

Reference Control B, n= 1 (made with acetonitrile)

Reference Control B, n= 2 (made with acetonitrile)

Reference Control B, n= 3 (made with acetonitrile)

Reference Control C, n= 1 (made with acetonitrile)

Reference Control C, n= 1 (made with distilled water)

Phenylacetaldehyde/Squaric Acid Diethyl Ester, n= 1

Chemical 1, n= 1

Chemical 2, n= 1

Chemical 3, n= 1

Chemical 4, n= 1

Chemical 5, n= 1

Reference Control C, n= 2 (made with acetonitrile)

Reference Control C, n= 2 (made with distilled water)

Phenylacetaldehyde/Squaric Acid Diethyl Ester, n= 2

Chemical 1, n= 2

Chemical 2, n= 2

Chemical 3, n= 2

Chemical 4, n= 2

Chemical 5, n= 2

Reference Control C, n= 3 (made with acetonitrile)

Reference Control C, n= 3 (made with distilled water)

Phenylacetaldehyde/Squaric Acid Diethyl Ester, n= 3

Chemical 1, n= 3

Chemical 2, n= 3

Chemical 3, n= 3

Chemical 4, n= 3

Chemical 5, n= 3

Reference Control B, n= 4 (made with acetonitrile)

Reference Control B, n= 5 (made with acetonitrile)

Reference Control B, n= 6 (made with acetonitrile)

Percent depletion for chemicals 1,2 and 3 is calculated based upon the mean NAC/NAL peak area of the Reference Control C which are prepared with acetonitrile.

Percent depletion for chemicals 4 and 5 is calculated based upon the mean NAC/NAL peak area of the Reference Controls C which are prepared with distilled water.