

HPLC METHODOLOGY MANUAL

DISTRIBUTED PHARMACEUTICAL ANALYSIS LABORATORY (DPAL)

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INTRODUCTION AND SCOPE

The goal of the Distributed Pharmaceutical Analysis Lab (DPAL) is to provide the highest quality analysis possible. However, it is important to acknowledge that as a coalition of research based academic laboratories, the level of quality assurance, maintenance and record keeping may not be equivalent to that of a commercial or government analytical laboratory. DPAL participants are expected to conduct all experiments in accordance with proper analytical methodology and report all results in a transparent manner.

DPAL procedures for quantifying the active ingredients in pharmaceutical dosage forms are based on monographs published in the United States Pharmacopeia (USP) or British Pharmacopeia (BP). Modifications are made to these methods to adjust for the resources available. For example, since the samples are single packages that do not contain a large number of tablets, DPAL analyzes individual drug tablets rather than pooled samples of 20-50 tablets.

LEGAL CONSIDERATIONS

Participants in the DPAL should be aware of legal issues related to pharmaceutical analysis. These legal considerations help protect all those involved in the DPAL including those being served and those in academic laboratories.

Poor quality medications are reported to the World Health Organization (WHO) Rapid Alert system and to the Medical Regulatory Authority (MRA) in the country where the drugs originated. Since the DPAL conducts single tablet analysis, assay results must be replicated on several samples before triggering a report to the MRA or WHO. If a participant laboratory identifies a substandard product, Professor Lieberman will assist them in reporting the medication to the appropriate authorities. The DPAL may need to run additional tests such as LC-MS and/or the MRA may want to analyze the samples themselves to support legal action. Therefore, samples and their packaging must be preserved carefully.

Due to the nature of the samples, secure data sharing and sample reporting is very important. The DPAL utilizes Open Science Framework (OSF) for communicating information with the intention of transparent data sharing among participants. Since information uploaded to OSF is primary data which is used in formal reports to regulatory agencies, it is imperative that the data is authentic and accurate. Therefore, folder access is restricted to only those individuals who actively report data or supervise operations. This policy is in place to prevent accidental or intentional editing by unauthorized users. For any questions regarding OSF, please reference the OSF Handbook found in the DPAL folder in OSF.

Due to the prevalence of counterfeit and improperly labeled products in developing world markets, it is possible that manufacturer information (manufacturer name, lot number, expiration date, etc.) stated on packaging materials is falsified or missing. At the recommendation of legal counsel at the University of Notre Dame, the DPAL requires that participants always reference samples as "stated to be manufactured by (company name)" for all communications about specific pharmaceutical products.

All product-specific information, including but not limited to photographs or metadata, that is posted in a public forum (poster session, news article, web site, social media platform, etc.) must adhere to the previously described wording ("stated to be manufactured by") for reference to the origin of the medication, unless the manufacturer has confirmed that they made the product. In addition, all comments about the products must be factual and non-inflammatory. Students must obtain permission from their instructor before posting anything regarding product information. It is the responsibility of the instructor to certify that the posting is, indeed, factual and noninflammatory.

DPAL ANALYSIS PROGRAM SET-UP

In order to provide quality analysis of pharmaceutical samples, several steps are necessary to ensure that the analytical methodology meets regulatory standards. The DPAL program is composed of distinct stages. The process begins with exploring a method based on published United States Pharmacopeia (USP) or British Pharmacopeia (BP) procedures. Once a functioning and efficient methodology has been established by one DPAL participant, other participants may use it if they can demonstrate system suitability to validate the method. Upon satisfactory completion of the system validation, the participant school will be sent pharmaceutical samples collected by our international partners to analyze.

Method Exploration

The appropriate USP or BP procedure is used as a starting point and assay conditions are optimized for the particular column and system. This process generally requires several hours in lab for a faculty member. Most assays require a standard 10-25 cm x 4.6 mm C18 column, HPLC grade methanol or acetonitrile as the organic solvent, buffer components, a pH meter and the appropriate reference standard. Samples must be syringe filtered. The DPAL can provide technical advice and funding assistance. Once a working method has been established, contact the DPAL to request expired samples for system suitability testing.

System Suitability

This stage occurs after the method conditions have been optimized and demonstrates that the instrument and developed method are in accordance with the standards laid out in USP <1226>. System suitability involves measuring the accuracy, precision, linearity, specificity, sensitivity, and limits of detection for the method. Detailed instructions for this stage can be found in the System Suitability Requirements Section. Demonstrating system suitability generally requires about 40 injections and makes for a good independent research or instrumental analysis team project. The DPAL will provide expired dosage forms for matrix recovery experiments. Data and results should be recorded in the System Suitability Spreadsheet Template (found in the DPAL folder on OSF). Other formats are acceptable as long as they contain all of the necessary information outlined in the System Suitability Requirements. The data from the system suitability experiments must be uploaded into OSF and reviewed before in-date pharmaceuticals are sent to the participant laboratory for analysis.

Sample Analysis

Running an assay consists of sample preparation, 1-4 injections of the sample, and analysis against an external reference. The external reference results are tracked as quality control (QC) samples and are run every five experimental samples. If the analytical metrics for a QC sample are out of the limits described in the protocol for a particular analyte, the results from all previous injections up until the last successful QC sample must be discarded and the samples rerun. (The experimental work can be easily completed in a 3-hour lab session, although there are logistical issues if many students are trying to use a small number of HPLCs).

Assay data and results for all pharmaceutical samples run during a session must be reported together in the appropriate template Excel spreadsheet (found in the Data Processing Templates folder in the DPAL project on OSF). The template spreadsheet includes blanks for all the

necessary data required for the DPAL. It is imperative that the spreadsheet be carefully completed to ensure proper data reporting. In addition, always maintain a current copy of the Control Chart (template found in the DPAL folder in OSF) for each analyte. Please see the OSF Handbook for full reporting procedures.

HPLC METHOD DEVELOPMENT

It is imperative that DPAL participants submit detailed and exact descriptions of the methodologies being employed. This ensures that accurate records are kept and allows other members of the DPAL to replicate the procedure. To submit method development information, please use the Excel template in the DPAL folder on OSF. Once the HPLC Method spreadsheet, System Suitability Requirements Information and a current Control Chart have been uploaded to OSF, the method will be reviewed and incorporated into the next update of the HPLC Methodology Manual.

Experimental Set-Up Information

- Analyte
- Instrument make and model
- Detector type, make and model
- λ Detector
- Column (Brand, Dimensions, Packing)
- Temperature (Room temperature? Column heated?)

Sample Information

- Concentration
- Solvent
- Reference material source, cost, purity
- Notes on sample prep (degradation, storage, sonication, mixing, etc.)
- Injection volume

Isocratic Methods

- Mobile phase (% water, % organic, buffer pH and conc., flow rate, additive conc.)
- Additive sources and costs
- How to make the buffer
- How to store the buffer
- Notes about the buffer
- Run times

Gradient Methods

- Mobile Phase A (% water, % organic, buffer pH and conc., additive conc.)
- Mobile Phase B (% water, % organic, buffer pH and conc., additive conc.)
- How to make the buffer
- How to store the buffer
- Notes about the buffer
- Description of gradient (run times, mobile phase ratios, flow rates and ramp types)

Column Washing

- Description of column washing protocol

SYSTEM SUITABILITY REQUIREMENTS

General Information

Information regarding personnel and the location of analysis is required for reporting purposes and is used as a means of contacting those involved with the project in the event that there are questions or concerns about the data. It is important to remember that student emails are deactivated upon graduation; therefore, the DPAL strongly encourages all student researchers to provide their personal email and have a faculty mentor that can oversee the program. For all of the following tests, the required information includes the analyst's name, date of completion, and mentor verification (initials) indicating that the experiment was properly conducted.

System Suitability experiments are separated into two parts depending on what material is used to prepare the solutions. Part One uses a certified reference standard to prepare solutions and include:

Normal Chromatograms

Include a chromatogram of a "normal" sample, analyzing the peak metrics as shown in the Analytical Metrics Section (page 9).

Establishing a Control Chart

Track metrics such as the peak shape, resolution, and integrated intensity of the known standard for each analyst and each day of operation. Use the Control Chart Excel Template found in the DPAL folder on OSF. Ensure that a current version is uploaded to OSF when submitting the System Suitability Report.

Precision

The relative standard deviation (RSD) for the integrated intensities of 6 consecutive injections of the known normal standard should be below 0.020 (2%).

Required Information:

- Intensity value measured
- RSD (in decimal units)

Linearity

Prepare and run at least five calibration standards over the concentration range of 5% to 200% of the standard. Calculate a regression line for the calibration data. The correlation coefficient, R, should be 0.98 or higher and the y intercept should be zero (within the error of measurement).

Required Information:

- Concentration values used
- Measurements for each point
- Calibration curve
- Slope and y-intercept (including units)
- R value

Accuracy & Range

Use volumetric techniques to accurately prepare overdosed (~150%), normal (~100%) and deficient (~35%) samples. Perform three replicate injections each of the overdosed sample (~150%), normal sample (100%), deficient sample (~35%), and a solvent blank (total of 12 determinations). Run the external standard after every 4 runs and check that the values of the integrated intensity for the external standard fall within 2% RSD. Use the average external standard signal to determine the concentrations of the overdosed, normal, deficient, and blank samples. The measured concentration of each sample should be within $\pm 2\%$ of its true concentration.

Required Information:

- True Concentration (T), Intensity, Measured Concentration (M) and (M-T)/T for all three of the runs for the overdosed sample, normal sample, deficient sample and solvent blank
- Listed intensities for the external standard (QC) samples
- RSD for QC samples

Limit of Detection (LOD) & Lower Limit of Quantification (LLOQ)

LOD and LLOQ determination are carried out using the slope of the calibration curve and the standard deviation of six low concentration samples. Best practice is to prepare the low concentration sample at about 2-3 times the expected LOD, which you can guess from your linearity plot. However if one measures the LOD and LLOQ, samples near the LOD or LLOQ limit should be run and their chromatograms shown in the report.

Required Information:

- Description of how LOD and LLOQ were measured and results of experiment
- (Or) Explanation of why this was not done

Part Two uses real dosage forms supplied by UND to prepare solutions and include:

Accuracy via Spike Recovery

A sample of a pharmaceutical dosage form (tablet or capsule) of the target drug should be prepared for analysis and a portion spiked with an extra 30% of the API. Calculate the % recovery of the spike. It should be within 90-110%.

Required Information:

- Description of how the sample was prepared and spiked
- % recovery of spike

Specificity

This can be demonstrated by showing that a spike can accurately be recovered from a degraded dosage form matrix. Stress the dosage form by baking a tablet for an hour at 60°C, then spike with an extra ~30% of the pure API and measure the spike recovery.

Required Information:

- Description of what was used as the matrix
- Description of how the matrix was spiked
- % recovery
- Chromatograms before and after the spike
- Calculated retention times and resolution for impurities

ANALYTICAL METRICS

Measuring Theoretical Plates

The DPAL recommends using the British Pharmacopeia method due to the simplicity of measuring peak width at half max height. Note, that this method will slightly underestimate column efficiency.

$$N = 5.54 \times \left(\frac{t_r}{W_{0.5}} \right)^2$$

t_r = retention time of the peak

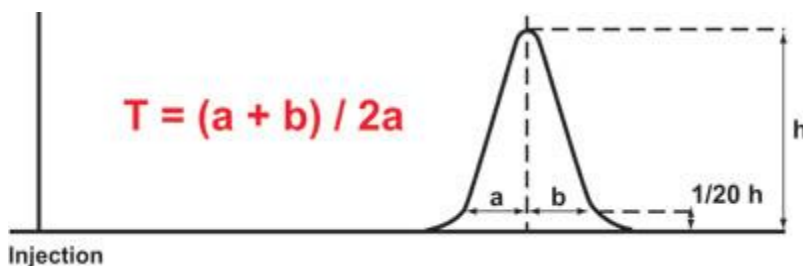
$W_{0.5}$ = width of peak at half the height

Measuring Resolution of Two Peaks

For peaks with tailing, the DPAL uses a formula with peak widths measured at 1/2 max height.

$$R_s = \frac{2(t_2 - t_1)}{1.7(W_{0.5,1} + W_{0.5,2})}$$

Measuring Tailing Factor



T = tailing factor

(measured at 5% of peak height) b = distance from the point at peak midpoint to the trailing edge

a = distance from the leading edge of the peak to the midpoint

Measuring Column Capacity Factor (Isocratic Methods Only)

$$k' = \frac{(t_r - t_0)}{t_0}$$

k' = column capacity factor

t_r = peak retention time

t_0 = dead volume of the column (measured by the elution time for the solvent front)

SAMPLE STORAGE AND TRACKING

The pharmaceuticals sent for analysis are forensic samples. Therefore, they must be stored in a way that does not promote degradation or contamination. It is imperative that thorough and accurate records of each sample are kept to ensure that the DPAL knows who performed what tests.

Samples must be stored in a cool, dark environment. The DPAL recommends storing samples in a plastic container with a tight lid in a refrigerator. Allow the samples to come to room temperature before working with them in order to prevent water from condensing on cold surfaces.

Each product sample in the batch you will receive consists of at least 3 tablets or capsules which will be shipped in a plastic bag labeled with a NDID tracking number (for example, the tracking number will be of the form "16-xxxx" for a sample cataloged in 2016). When tests are run on individual tablets or capsules, label them as "16-xxxxa, b, c". Students should sign out samples for analysis and use the NDID tracking number in their records and chromatogram labels.

SAMPLE PREPARATION

External Standards

External calibration standards are created from analytical grade reagents that are traceable to USP or BP standards. The standard should include a certificate of analysis, and the reagent purity, protonation state, and hydration state should all be taken into account when calculating final concentrations (see the Excel template for guidance). Store dry standards as directed on the bottle (most must be kept cold).

Paracetamol Standard	Sigma-Aldrich	PHR1005 – 1G	\$57.00
Ampicillin Trihydrate Standard	Sigma-Aldrich	PHR1393 – 1G	\$61.70
Amoxicillin Trihydrate Standard	Sigma-Aldrich	PHR1127 – 1G	\$57.00
Potassium Clavulanate Standard	Sigma-Aldrich	33454 – 100MG	\$129.50
Ciprofloxacin Standard	Sigma-Aldrich	PHR1167 – 1G	\$57.00
Azithromycin Standard	Sigma-Aldrich	PHR1088 – 1G	\$62.70

The "known" API standard should contain about 0.5 mg/mL of the active pharmaceutical ingredient (API) being analyzed (for amoxycylav analysis the standard should contain 0.5 mg/ml of amoxicillin plus 0.2 mg/ml of clavulanate). The precise concentration is not important, but the exact concentration must be known (use the analytical balance and volumetric fluid measurements). For most analytical balances, a minimum mass of 50.0 mg is required for suitable accuracy. This external standard will be used to determine the sample concentrations.

It is best practice to make a fresh standard sample every time it is needed. However, this protocol is expensive and time consuming. If a participant wants to reuse standards, the data and results of at least one experiment must be conducted in which the reused standard was assayed against a fresh one (it would be a good, short research project to run periodic chromatograms for a standard sample that is left out at room temperature or under refrigeration for several days).

For system suitability, you will need the known standard, five calibration standards; a set of normal, overdosed, and deficient "unknowns"; and a dosage form of the product that will be used for a spike-recovery experiment. The HPLC experiment only requires 20 µL per injection, but in order to get accurate dilutions, one must prepare the solutions using volumetric glassware. Excess solutions may be aliquoted into Eppendorf tubes and frozen for up to 1 month.

Calibration standards should span the range from 5% to 200% of the expected API concentration in the experimental samples. At least 5 standards should be used to construct the calibration curve. For example, use 5%, 20%, 80%, 120%, and 200% to establish linearity. A calibration curve generated on one day cannot be used to assay concentrations of samples run on another day. Since it takes 5 runs to do the calibration curve, the DPAL prefers to establish linearity, and then use a single-point external standard to assay concentrations of unknown samples.

Prepare a "normal" unknown sample in the 95-105% range, an "overdosed" sample in the 140-160% range, and a "deficient" sample in the 20-50% range. Also, prepare a method blank, which is nominally 0%. For the spike recovery experiment, a dosage form (tablet or capsule) is

required, which DPAL can send to participants. Prepare the sample as described below under "pharmaceutical dosage forms," weighing out at least 50 mg of the powdered tablet. Weigh out 25 mg of the API and add, then prepare and filter the sample as described below; the nominal concentration should be around 150% of the expected API content (calculate it exactly).

Pharmaceutical Dosage Forms

Samples for analysis should contain about 0.5 mg/mL of the API (0.5 mg/ml of amoxicillin and 0.2 mg/mL of clavulanate for amoxycrav analysis) unless otherwise stated in the method. This concentration may need to be adjusted to ensure that the samples are in the linear range for a particular system.

Accurately weigh a tablet or the contents of a gel capsule and take a portion of the powder that will give a 0.5 mg/ml solution of the API when diluted to volume. For capsules, weigh the contents of a gel cap by difference. Weigh the entire capsule, then remove the powder and use a stream of air to blow out any remaining powder, finally reweigh the empty capsule. For tablets, the entire tablet should be weighed and then crushed with a mortar and pestle. The powder from a tablet or capsule should be well mixed. For preparation of the analytical sample, weigh out at least 50 mg of powder on an analytical balance. The remaining powder should be labeled and frozen for storage. The label should include the date, API name, sample number, and analyst's initials.

For example, the total contents of an amoxicillin capsule with a nominal dose of 500 mg amoxicillin might weigh 627 mg due to excipients. To prepare a 0.5 mg/mL solution, a portion of roughly 63 mg would be accurately weighed and dissolved in 100 mL of solvent. Samples should be thoroughly mixed (by 5 minutes of sonication or 5 minutes on a magnetic stirrer) and an additional 2 minutes of hand shaking and inversion of the volumetric flask. All samples must be filtered through a fresh 0.45-micron syringe filter to remove particulates that might clog the HPLC column. The DPAL recommends filtering about 1 mL of the sample into an autosampler vial, discarding the first drops of filtrate.

COLUMN STORAGE, CONDITIONING AND WASHING

For 4.6 mm ID columns, typical column volumes are 4.2 mL for a 25 cm column, 2.5 mL for a 15 cm column, and 1.7 mL for a 10 cm column.

Column Storage

50% organic solvent (methanol or acetonitrile):50% water. It is good practice to label individual columns so students can record which column was used to produce each assay. Many columns are pre-labeled with a unique ID to facilitate tracking the usage.

Conditioning the Column

If a buffer solution is run through the column while it is full of 50% methanol, the buffer salts may precipitate and clog the column. Condition the column by running 95% water:5% methanol (initial water: organic ratio) for 5 column volumes. Use a flow rate that gives back pressures in the 1500-2400 psi range. Next run 95% buffer:5% methanol (initial conditions, with buffer) for 10 column volumes. Do a blank run and check that the background is clean. Conditioning will take 1-2 hours, during which time samples can be prepared.

Washing the Column

Do not leave the column with low-organic buffer solution (<30% methanol or acetonitrile) in it, because bacterial growth will occur. If the column needs to be stored for more than 10 hours, protect the column by washing it. Run 5 column volumes of 100% water to remove traces of buffer salts, then run 5 column volumes of 50% methanol:50% water (or 50% acetonitrile:50% water if the method uses acetonitrile). The column can be left on the HPLC or removed and capped for storage.

SAMPLE ASSAY AND QUALITY CONTROL PROCEDURES

Reproducibility

External calibration standards are created from analytical grade reagents as directed in section 4. Five injections of the external standard must show a peak area within 2% relative standard deviation (RSD), and the range of retention times must be within 0.5 minutes, before samples are assayed.

Control Chart

Each time the method is performed, record the date and the retention time and integrated intensity of the 5th external calibration standard. Also record changes to the method (for example, use of a new column or different batch of buffer). The intensities and retention times should be plotted on a graph. If the intensities or retention times vary outside the control limits, the system suitability is in question. Control chart problems are usually caused by clogged columns or instrumental problems such as bad valve blocks or buffer salt precipitation. These issues must be resolved before carrying on with assays.

Quality Check

After every five unknown sample runs, the standard is injected as a quality check and it must assay within 2% RSD of the 5 initial injections and be within the 0.5 minute time range of the initial injections. If a quality check fails, data after the last passed quality check cannot be used.

Replicate Samples

Typically, one pill from each package is analyzed. If a sample fails analysis (assay value <90% or >110% of stated API content) then two new samples are prepared independently from the remaining powdered pill material and re-assayed. Report all three assays and calculate their average and standard deviation. If the average also fails and you want to measure pill-to-pill variability in the packet, two more tablets may be assayed. The spreadsheet posted at the DPAL website has macros set up to do the analytical calculations – but make sure you understand how those macros work!

HPLC001p19										6/5/14																													
STD #		Amox Peak Area		Clav Peak Area		STD Conc (mg/mL)		Mass Amox (g)		Volume (mL)		Used (mL)		Final Vol (mL)		Purity		Conc (mg/mL)																					
1		55211		18136				0.0213		100		100		100		0.987		0.1831408				Expressed as Amoxicillin																	
2		55263		18235																																			
3		54942		18668				Mass Clav (g)		Volume (mL)		Used (mL)		Final Vol (mL)		Purity		Conc (mg/mL)																					
4		55063		18107				0.0118		10		5		100		0.999		0.04947765				Expressed as Clavulanic Acid																	
5		55071		18189																																			
AVG		55130		18267																																			
SD		128.027341		229.4540286																																			
%RSD		0.23231236		1.256331136																																			
Sample						Pill Total Mass (g)		Sample Mass (g)		Volume (mL)		Amox Dosage (mg)		Clav Dosage (mg)		mg Amox		mg Clav		%Error Amox		% Error Clav																	
13-0147		42991		11992		1.1207		0.0293		100		500		125		546.45		124.23823		9.29		-0.6094199																	
13-0131		38724		12120		1.0931		0.0272		100		500		125		517.16		131.73161		3.49		5.36528948																	
13-0128 Pill 2		27969		0		1.1259		0.0264		100		500		125		396.39		0		-20.72		-100																	
13-0128 Pill 3		38896		12374		1.1067		0.0269		100		500		125		531.79		137.889		6.36		10.311966																	
13-0153		39348		12554		1.1115		0.0279		100		500		125		520.93		135.46567		4.19		8.37253743																	
Cal. Check_1		56357		18258																																			
Avg		55317.8333		18265.5																																			
SD		521.805487		205.2985631																																			
%RSD		0.9432862		1.123969029																																			
Sample						Pill Total Mass (g)		Sample Mass (g)		Volume (mL)		Amox Dosage (mg)		Clav Dosage (mg)		mg Amox		mg Clav		%Error Amox		% Error Clav																	
13-0148		43485		15183		1.0299		0.0301		100		500		125		483.08		140.71099		-3.38		12.5687928																	
13-0136 Pill 2		27769		0		1.1144		0.0274		100		500		125		375.32		0		-24.94		-100																	
13-0138 Pill 1		33991		724		1.1067		0.0261		100		500		125		478.97		8.3151452		-4.21		-93.347884																	
13-0138 Pill 3		33788		0		1.1289		0.0319		100		500		125		397.36		0		-20.53		-100																	
Cal. Check_2		55248		18046																																			
Avg		55307.8571		18234.14286																																			
SD		477.071773		204.9531886																																			
%RSD		0.86257504		1.124007804																																			

A copy of this Excel spreadsheet is posted on the DPAL site.

HPLC Method Development

Analyte: _____

Instrument: _____

Detector: _____

$\lambda_{\text{detector}}$ _____

Column used: _____

Brand name

Column dimensions

Packing

Column temperature: RT (no column heater)

Column heater, set to _____

Sample (pick something in the linear range) _____

conc in what solvent?

Notes on sample prep

For isocratic methods:

Mobile phase: _____

% water

% organic

buffer, pH, and conc. additive concs

How do you make the buffer?

How do you store the buffer? Any notes, safety concerns?

For gradient methods:

Mobile phase A: _____

% water

% organic

buffer, pH, and conc. additive concs

Mobile phase B: _____

% water

% organic

buffer, pH, and conc. additive concs

How do you make the buffer?

How do you store the buffer? Any notes, safety concerns?

Describe the gradient:

Column washing: After use, run 5 column volumes of 100% water to remove traces of buffer salts, then run 5 column volumes of 50% methanol:50% water (or 50% acetonitrile:50% water if your method uses acetonitrile).

HPLC Method Validation

Analyte: _____

The goal is to establish that a standard method, when run on your instrument and with your reagents, satisfies analytical metrics. These directions are specific for HPLC using an external standard with a fixed-wavelength UV-Vis or diode-array detector. Separate this checklist and the method validation results from the routine assay results. It is recommended that records be kept both electronically and in hard copy. A minimum of 24 injections will be required, past validations have taken 30-70 hours.

- ☐ Precision: The relative standard deviation (RSD) for the integrated intensities of 6 consecutive injections of the known standard should be below 2%.
- ☐ Establish control chart: Set up a record keeping page to track metrics such as the integrated intensity of the known standard for different analysts and days of operation. The control limits for the integrated intensity of the signal will be set at the average value from the precision measurement $\pm 10\%$ (eg, if the average was 20,000 units, the control limits would be $20,000 \pm 2,000$). Place the control chart at the front of the binder.
- ☐ Linearity: Prepare and run a least five calibration standards over the concentration range of 5% to 200%. Calculate a regression line for the calibration data, including correlation coefficient, y-intercept, slope, and residual sum of squares. The correlation coefficient should be 0.98 or better and the y-intercept should be zero within error of measurement.
- ☐ Accuracy and range: Perform three replication assays each of the overdosed sample, the normal sample, the deficient sample, and a solvent blank (total of 12 determinations). Run the external standard after every 5 runs and check that the values of the integrated intensity for the external standard fall within 2% RSD. Use the average external standard signal to determine the concentrations of the overdosed, normal, deficient, and blank samples. The measured concentration of each sample should be within 2% of its true concentration.
- ☐ Accuracy via spike recovery: If available, a sample of a pharmaceutical dosage form (tablet or capsule) of the target drug should be prepared for analysis and a portion spiked with an extra 30% of the API. Calculate the % recovery of the spike; it should be within 90-110%.
- ☐ Specificity: This can be demonstrated by showing that a spike can accurately be recovered from a dosage form matrix (the test in the “accuracy via spike recovery” section). A more robust demonstration would be to stress the dosage form (eg, by baking the tablet or powder for an hour at 60°C), then use that as the matrix for a spike recovery experiment.
- ☐ Optional: LOD and LLOQ: LOD or LLOQ determination is carried out by using the slope of the calibration curve and the SD of low concentration samples. Best practice is to prepare a sample at about 2-3 times the expected LOD; you could also use the SD for blank runs. However you measure the LOD and LLOQ, samples near the LOD or LLOQ limit should be run and their chromatograms shown in the report.

Control Chart

Reference standard: _____

API name Manufacturer Lot number Assay %

How to make the standard sample: _____

conc in what solvent?

how stable is the sample at RT? _____
hours initials, notebook reference

How stable is the sample at 0°C? _____
hours initials, notebook reference

Precision measurement: _____

average of 6 intensity measurements initials, notebook reference

The control limits are the average value from your precision measurement with a $\pm 10\%$ margin (eg, if the average was 20,000 units, the control limits would be 20,000 \pm 2,000).

Date	Analyst	Retention time(s)	Resolution (if there are two analytes)	Integrated peak intensity	Tailing factor	# Theoretical plates

METHODS

Methods Validated at Notre Dame:

Acetaminophen (Paracetamol)
Albendazole
Amoxicillin and Amoxicillin/Clavulanate
Ampicillin
Artemether/Lumefantrine
Benzyl Penicillin (Penicillin G)
Carboplatin (In Progress)
Ceftriaxone
Ciprofloxacin
Cisplatin
Doxorubicin
Doxycycline
Enalapril
Gentamicin (In Progress)
Losartan
Metformin
Methotrexate
Omeprazole
Oxaliplatin
Oxytocin
Quinine

Methods Validated at DPAL Participants:

Acetaminophen (Maryville College)
Ampicillin/Cloxacillin (IU-Indianapolis and Niagara University)
Azithromycin (Dublin Institute of Technology)
Albendazole (Grand View University)
Doxycycline (US Coast Guard Academy)
Levofloxacin (Geneva College)
Metformin (Niagara University and Murdoch University)
Ofloxacin (Indiana University-Bloomington)

Methods Yet to be Validated (USP or Schools Methods Shown):

Cefuroxime (Grand View Started)
Cephalexin (Virginia Wesleyan Started)
Propofol
Sildenafil
Sulfamethoxazole/Trimethoprim (Newman and Southern Oregon Started)

ACETAMINOPHEN (PARACETAMOL)

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 243 nm

Column: Symmetry Shield 150 x 4.6 mm, C18 packing, 3.5 μ m particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.1 mg/mL

Sample Solvent: Mobile phase. Solubility can be an issue with acetaminophen so it may be helpful to dissolve the powder in methanol and dilute to volume with water rather than dissolve with the mobile phase directly.

Mobile Phase: 3:1 mixture of water and methanol

Flow Rate: 1.0 mL/min

Sample Injection Volume: 20 μ L

Run Time: 6 minutes

Column Washing: 10 column volumes of methanol. Slowly bring to 100% water and rinse for 10 column volumes before bringing to storage conditions.

Column Storage: 50:50 methanol-water.

Analytical Metrics for Acetaminophen

Column Efficiency >1000 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Acetaminophen Chromatogram



ALBENDAZOLE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 254 nm

Column: XTerra 150 x 4.6 mm, C18 column, 3.5 μ m particle size

Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.5 mg/mL

Sample Solvent: Acidified methanol prepared by adding 1 mL of sulfuric acid to 99 mL of methanol. Sample prep involves dissolving 50 mg of powder in 5 mL of acidic methanol and 25 mL methanol in a 50 mL volumetric flask. Sonicate for 5 minutes and bring to volume with methanol.

Mobile Phase: Dissolve 0.50 g of monobasic ammonium phosphate in 400 mL of water. Add 600 mL of methanol, mix and filter. Bring pH to 1.7-2.0 by using a drop or two of sulfuric acid.

Flow Rate: 1.0 mL/min

Injection Volume: 15 μ L

Run Time: 7 minutes

Column Washing: 10 column volumes of storage solution

Column Storage: 50:50 methanol-water.

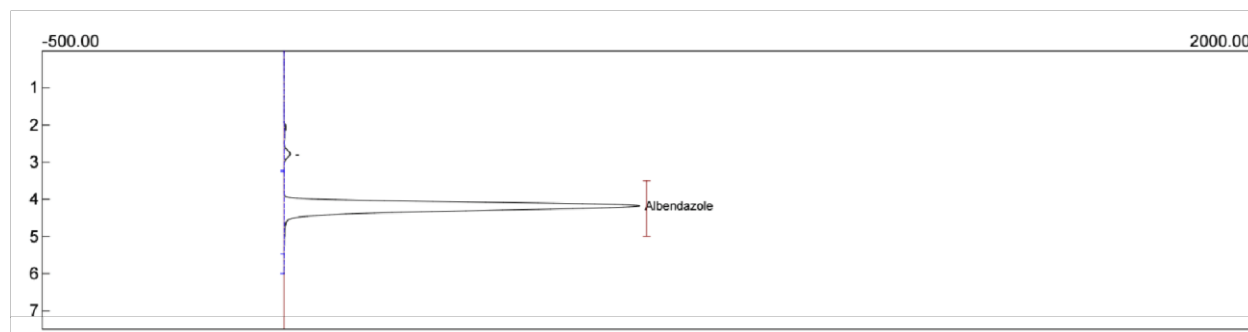
Analytical Metrics

Column Efficiency >2000 theoretical plates

Tailing Factor <1.5

RSD for Replicate Injections <0.3%

Typical Albendazole Chromatogram



AMOXICILLIN AND AMOXICILLIN/CLAVULANATE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 220 nm

Column: Symmetry 100 x 4.6 mm, C18 column, 5 µm particle size and 100Å

Column Temperature: Room temperature

Gradient Method

Sample Concentration: 0.5 mg/mL

Amoxicillin Sample Solvent: Water

Amoxyclav Sample Solvent: Water, samples should be used within 6 hours of preparation.

Mobile Phase A: 100% methanol

Mobile Phase B: 20 mM monosodium phosphate buffer, pH 4.4

Flow Rate: 1.0 mL/min

Sample Injection Volume: 20 µL

Gradient Description:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Ramp
0	5	95	Equilibrating
0.5	5	95	None
5	30	70	Linear
7	90	10	Linear
8	90	10	None
8.5	25	75	Linear
10	10	90	Linear
11	5	95	Linear
12	5	95	None

Column Washing: 5 column volumes of 95:5 water-methanol, 5 column volumes of 50:50 water-methanol, 5 column volumes of 5:95% water-methanol, then 5 column storage solution.

Column Storage: 60: 40 methanol-water.

Analytical Metrics for Amoxicillin

Column Efficiency >1700 theoretical plates

Tailing Factor <2.5

RSD for Replicate Injections <2.0%

Analytical Metrics for Amoxy-clav

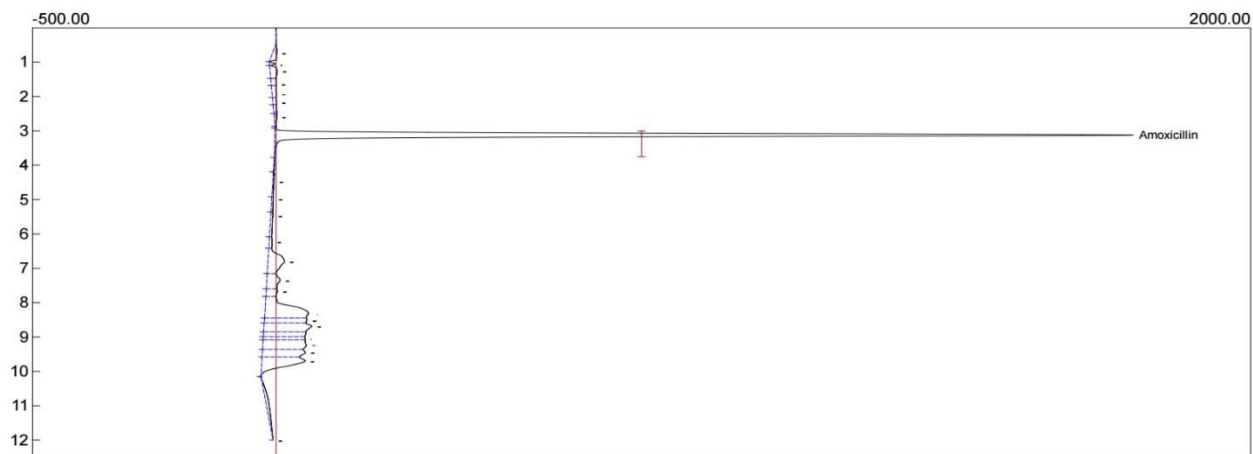
Resolution between the amoxicillin and clavulanate peaks > 3.5

Column Efficiency >550 theoretical plates

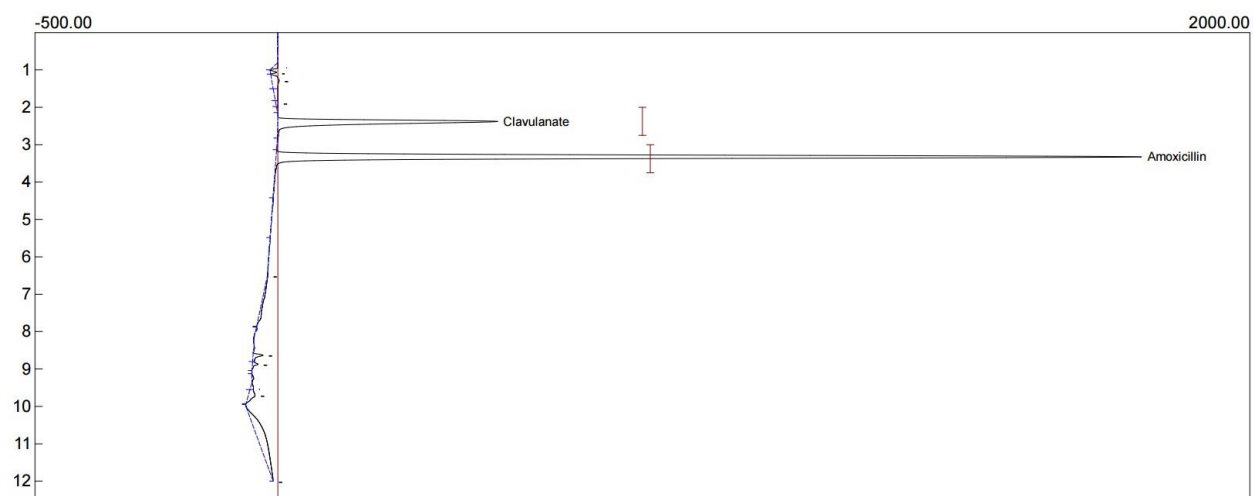
Tailing Factor <1.5

RSD for Replicate Injections <2.0% for both APIs

Typical Amoxicillin Chromatogram



Typical Amoxy-clav Chromatogram



AMPICILLIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 230 nm

Column: XBridge 50 x 3 mm, C18 column, with 5 μ m particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.5 mg/mL

Sample Solvent: Water

Buffer: 2.7 g sodium phosphate monobasic in 1000 mL adjust to pH 4.4 ± 0.1

Mobile Phase: Methanol and Buffer (20:80)

Sample Injection Volume: 40 μ L

Flow Rate: 1 mL/min

Run Time: 7 min

Column Washing: 5 column volumes of 100% water to remove traces of buffer salts, then run 5 column volumes of storage solution.

Column Storage: 50:50 methanol-water.

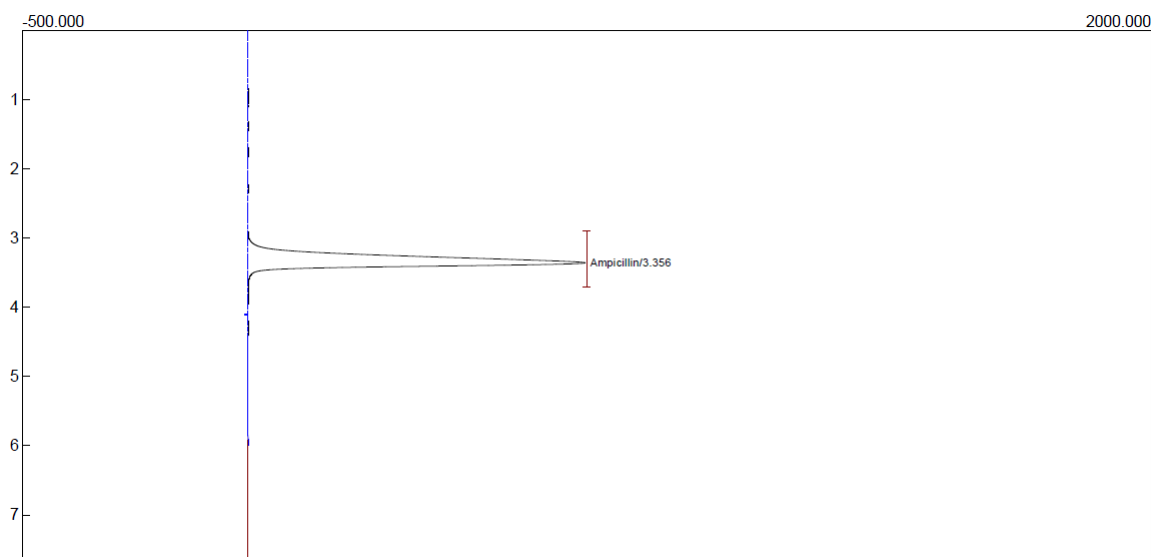
Analytical Metrics for Ampicillin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <1.4

RSD for Replicate Injections <2.0%

Typical Ampicillin Chromatogram



AMPICILLIN-CLOXACILLIN (Niagara University Method Shown)

Instrument: Agilent 1200 High Performance Liquid Chromatograph

Detector: Agilent VWD G1314B Model

Analytical Wavelength: 220 nm

Column: Agilent XDB 150 x 4.6 mm, C18 column, with 5 µm particle size

Column Temperature: Room temperature

Gradient Method

Sample Concentration: 0.1 mg/mL of both APIs

Sample Solvent: Water

Mobile Phase A: 3.3 mL phosphoric acid with 800 mL water. Bring to pH 5.0 with concentrated sodium hydroxide. Dilute to 1 L with water.

Mobile Phase B: Acetonitrile

Sample Injection Volume: 5 µL

Flow Rate: 1.0 mL/min

Gradient Description:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Ramp
0	85	15	Equilibrating
1	85	15	Linear
15	70	30	Linear
20	85	15	Equilibrating

Column Washing: Ramp over 3 minutes from 85% buffer 15% acetonitrile to 30% buffer 70% acetonitrile. Hold this concentration for 20 minutes. Ramp over 3 min to 85% buffer 15% acetonitrile. Rinse 15 minutes with 85% water 15% acetonitrile.

Column Storage: 15:85 acetonitrile-water.

Analytical Metrics for Ampicillin-Cloxacillin

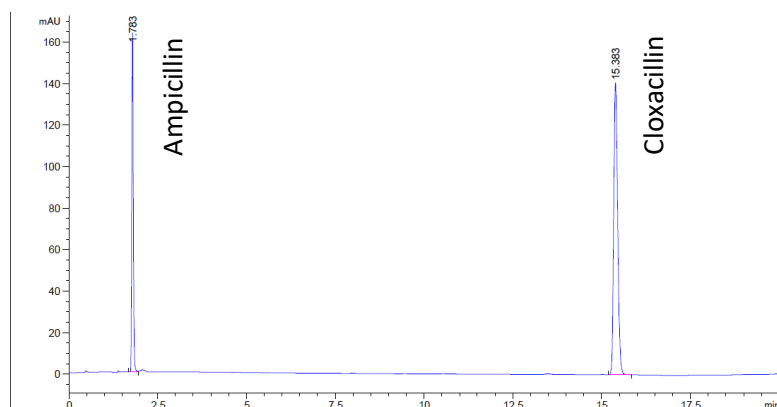
Resolution between ampicillin and cloxacillin peaks >10

Column Efficiency >3000 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Ampicillin-Cloxacillin Chromatogram



ARTEMETHER-LUMEFANTRINE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 210 nm

Column: XTerra 150 x 4.6 mm, C18 column, with 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 10 mg/mL artemether and 0.5 mg/mL lumefantrine

Sample Solvent: Mobile Phase

Buffer: 6.0 g monosodium phosphate in 1000 mL adjust to pH 2.4

Mobile Phase: Methanol and Buffer (84:16)

Sample Injection Volume: 20 µL

Flow Rate: 1.0 mL/min

Run Time: 10 min

Column Washing: 10 column volumes of 95:5 water-methanol to remove traces of buffer salts, then run 10 column volumes of storage solution.

Column Storage: 50:50 methanol-water.

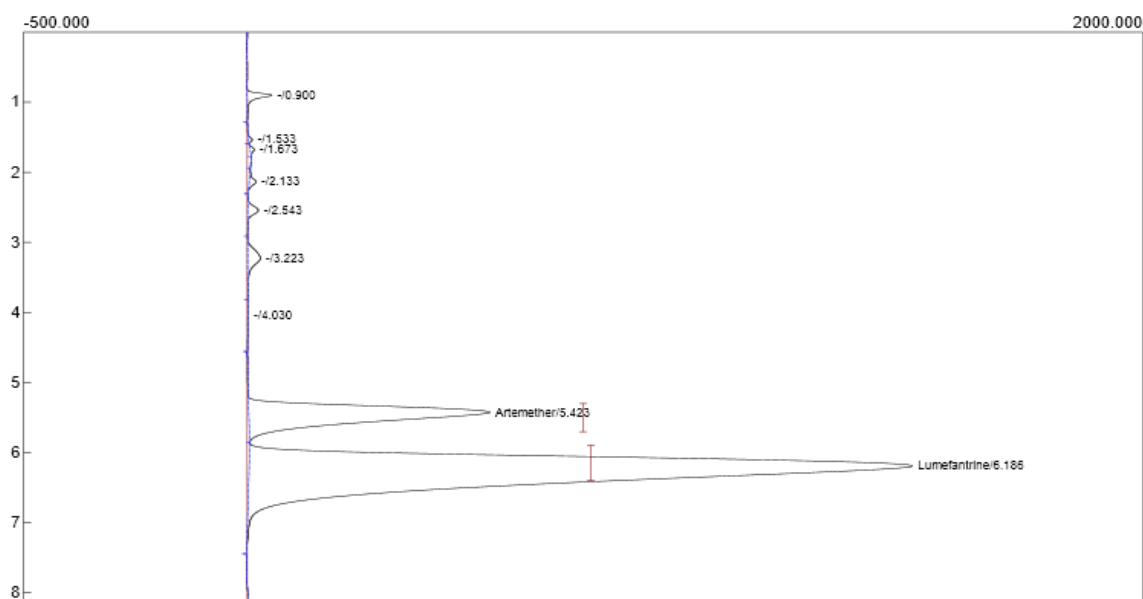
Analytical Metrics for Artemether-Lumefantrine

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <1.4

RSD for Replicate Injections <2.0%

Typical Artemether-Lumefantrine Chromatogram



AZITHROMYCIN (Dublin Institute of Technology's Method Shown)

Instrument: Agilent 1200 Series High Performance Liquid Chromatograph

Detector: Agilent Triple Quadrupole 6410 Detector

Analytical Wavelength: Transition (m/z): 749.5 to 591.4

Column: Kinetex 50 x 2.1 mm, C18 column, with 2.6 µm particle size and 100 Å

Column Temperature: 35 °C

Gradient Method

Sample Concentration: 0.004 mg/mL

Mobile Phase A: Water with 0.1% formic acid

Mobile Phase B: Acetonitrile with 0.1% formic acid

Sample Diluent: Acetonitrile

Sample Injection Volume: 1 µL

Flow Rate: 0.3 mL/min

Run Time: 8 minutes

Gradient Description:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Ramp
0	90	10	Equilibrating
2	90	10	None
4	0	100	Linear
5	0	100	None
5.01	90	10	Linear
8	90	10	Equilibrating

Column Washing: Wash with storage solution for 10 column volumes.

Column Storage: Store in 90:10 acetonitrile-water

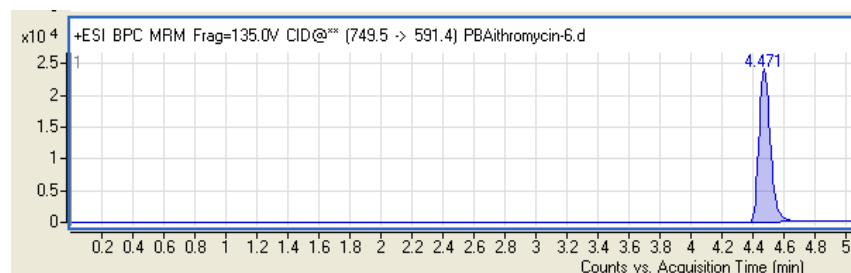
Analytical Metrics for Azithromycin

Column Efficiency >1000 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Azithromycin Chromatogram



BENZYL PENICILLIN (PENICILLIN G)

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 220 nm

Column: Symmetry 100 x 4.6 mm, C18 packing, 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 1.0 mg/mL

Sample Solvent: 5 mL of methanol and 10 mL acetonitrile then sonicate for 3-5 minutes before diluting to 50 mL with water

Mobile Phase: 10 mM monobasic potassium phosphate (pH 6) and methanol (40:60)

Flow Rate: 1.0 mL/min

Sample Injection Volume: 10 µL

Run Time: 5 minutes

Column Washing: Rinse with 10 column volumes of 95:5 water-methanol to remove traces of buffer salts, then 10:90 water-methanol for 5 column volumes, run 5 column volumes of storage solution.

Column Storage: 50:50 methanol-water.

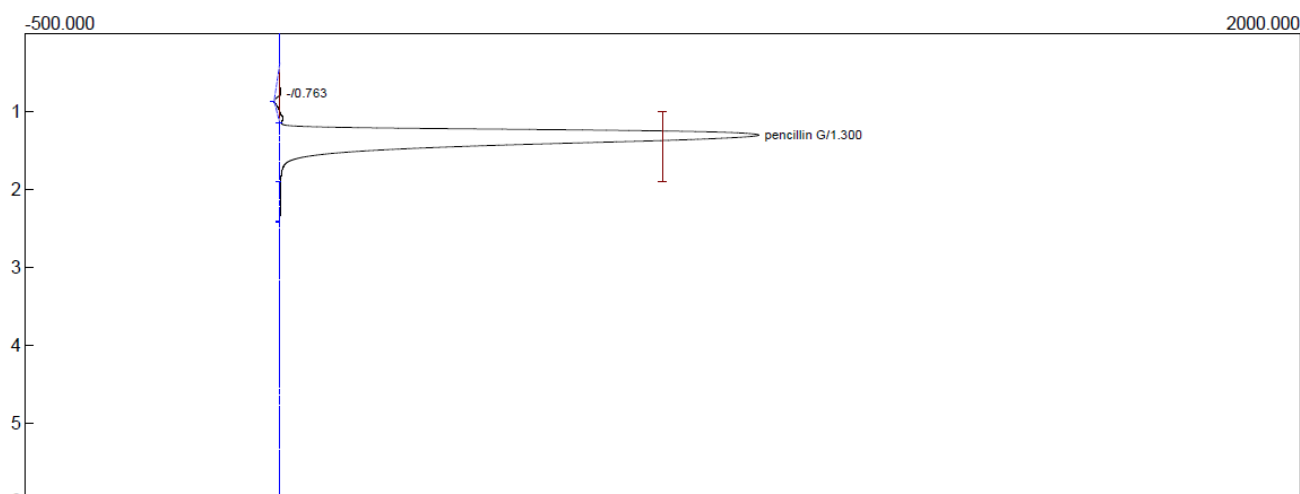
Analytical Metrics for Benzylpenicillin

Column Efficiency >1000 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Benzylpenicillin Chromatogram



CARBOPLATIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 230 nm

Column: Waters Spherisorb 250 x 4.0 mm, NH₂ (L8) packing, 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 1.0 mg/mL

Sample Solvent: Water

Mobile Phase: Acetonitrile and water (87:13)

Flow Rate: 2.0 mL/min

Sample Injection Volume: 20 µL

Run Time: 16 minutes

Column Washing: Rinse with 10 column volumes water then run 5 column volumes of storage solution.

Column Storage: 50:50 acetonitrile-water.

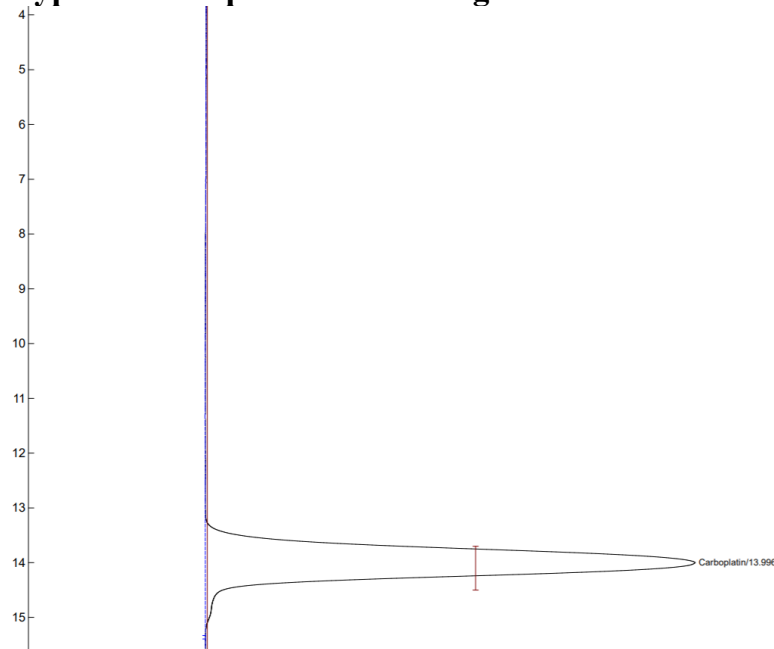
Analytical Metrics for Carboplatin

Column Efficiency >2500 theoretical plates

Tailing Factor <2.5

RSD for Replicate Injections <2.0%

Typical Carboplatin Chromatogram



CEFTRIAXONE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 240 nm

Column: Kinetex 250 x 4.6 mm, C18 column, 5 μ m particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.2 mg/mL

Sample Solvent: Mobile phase

Buffer A: 13.6 g of dibasic potassium phosphate and 4.0 g of monobasic potassium phosphate in 1000 mL water. Adjust to pH 7.0 with phosphoric acid or 10 N KOH

Buffer B: 25.8 g of sodium citrate in 500 mL of water, adjust to pH 5 with citric acid solution (1 in 5) and bring to 1000 mL

Mobile Phase: 3.2 g of tetraheptylammonium bromide in 400 mL of acetonitrile, 44 mL of Buffer A, 4 mL of Buffer B, and bring to 1000 mL with water

Sample Injection Volume: 20 μ L

Flow Rate: 1.0 mL/min

Run Time: 10 minutes

Column Washing: Rinse with 10 column volumes of 90:10 water-acetonitrile then wash with 10 column volumes of storage solution.

Column Storage: 50:50 acetonitrile-water.

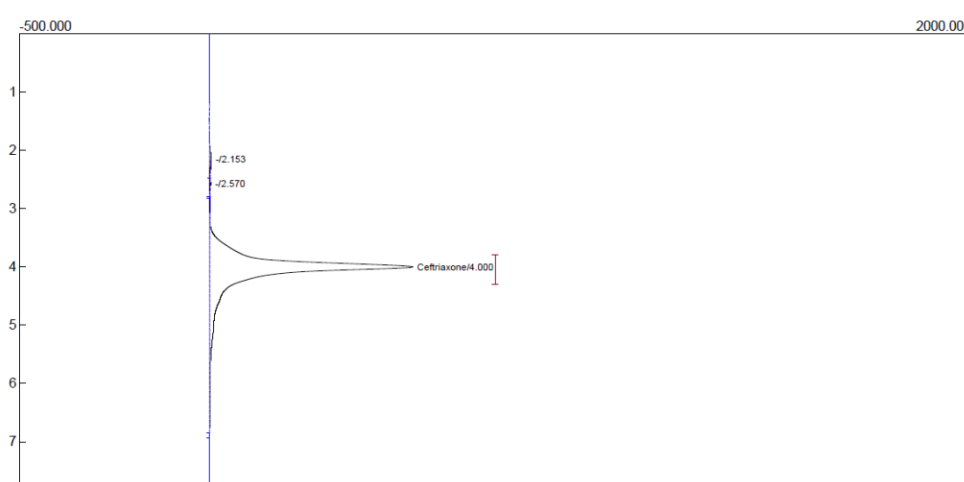
Analytical Metrics for Ceftriaxone

Column Efficiency >1500 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Ceftriaxone Chromatogram



CEFUROXIME (USP Method Shown)

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 278 nm

Column: Brand 250 x 4.6 mm, C1 column, with 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.20 mg/mL

Sample Solvent: Methanol then diluted with 0.2 M monobasic ammonium phosphate

Mobile Phase: Methanol and 0.2 M monobasic ammonium phosphate (1:3)

Sample Injection Volume: 10 µL

Flow Rate: 1.5 mL/min

Run Time:

Column Washing:

Column Storage:

Analytical Metrics for Cefuroxime

Column Efficiency >3000 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.0%

Typical Cefuroxime Chromatogram

CEPHALEXIN (USP Method Shown)

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 254 nm

Column: 250 x 4.6 mm, C18 column

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.4 mg/mL

Sample Solvent: Water

Mobile Phase: 0.985 g/L of sodium-1-pentanesulfonate in mixture of acetonitrile, methanol, triethylamine, and water (20:10:3:170) adjusted to pH 3.0 with phosphoric acid

Sample Injection Volume: 20 µL

Flow Rate: 1.5 mL/min

Run Time:

Column Washing:

Column Storage:

Analytical Metrics for Cephalexin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.0%

Typical Cephalexin Chromatogram

Instrument: Waters e2695 High Performance Liquid Chromatograph
Detector: Waters 2487 Dual-Wavelength Absorbance Detector
Analytical Wavelength: 278 nm
Column: XTerra 100 x 4.6 mm, C18 column, with 3.5 µm particle size
Column Temperature: Room temperature

Sample Concentration: 0.5 mg/mL
Sample Solvent: Mobile phase
Buffer: Dilute 2.9 mL of phosphoric acid in water to 1000 mL, adjust to pH 3.0 with triethylamine
Mobile Phase: Acetonitrile and Buffer (135:865)
Sample Injection Volume: 10 µL
Flow Rate: 1.5 mL/min
Run Time: 12 min
Column Washing: Wash with 10 column volumes of 100% water to remove any trace buffer salts and then wash with 10 column volumes of storage solution.
Column Storage: 50:50 acetonitrile-water.

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates
Tailing Factor <4.0
RSD for Replicate Injections <2.0%

CISPLATIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 230 nm

Column: Luna 250 x 4.6 mm, C8 column, 100 Å

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 1 mg/mL

Sample Solvent: 0.9% saline (NaCl) solution

Mobile Phase: Methanol and water (10:90)

Sample Injection Volume: 40 µL

Flow Rate: 1.0 mL/min

Run Time: 6 min

Column Washing: Wash with storage solution.

Column Storage: 50:50 methanol-water.

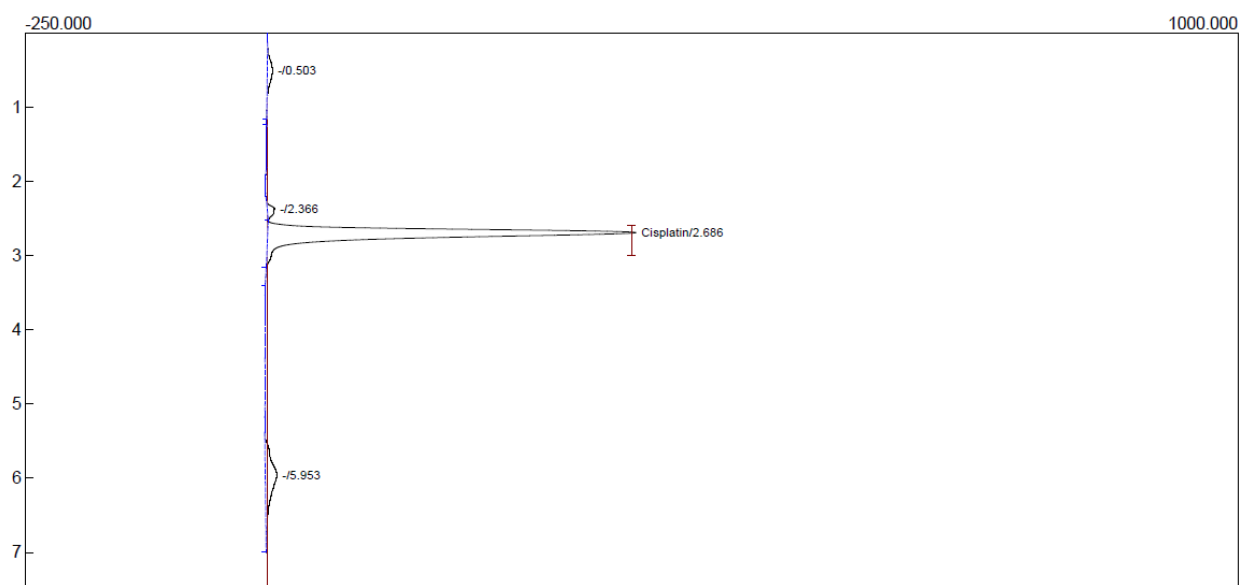
Analytical Metrics for Cisplatin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.0%

Typical Cisplatin Chromatogram



DOXORUBICIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 254 nm

Column: Spherisorb 250 x 4.6 mm, C18 column, with 5 μ m particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.1 mg/mL

Sample Solvent: Mobile phase

Mobile Phase: 1 g/L sodium lauryl sulfate dissolved in 54% water, 29% acetonitrile, 17% methanol, and 0.2% phosphoric acid brought to pH 3.6 \pm 0.1 using 2 M sodium hydroxide.

Sample Injection Volume: 20 μ L

Flow Rate: 1.5 mL/min

Run Time: 10 min

Column Washing: Wash with storage solution.

Column Storage: 50:30:20 water-acetonitrile-methanol.

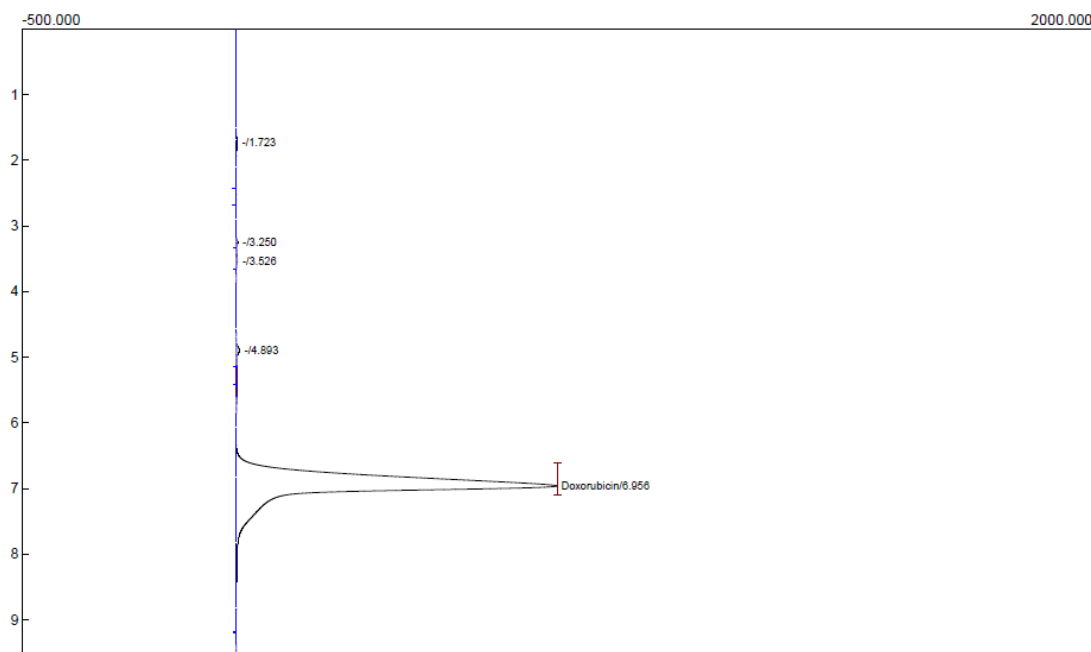
Analytical Metrics for Doxorubicin

Column Efficiency >2250 theoretical plates

Tailing Factor >0.7 and <1.2

RSD for Replicate Injections <1.0%

Typical Doxorubicin Chromatogram



DOXYCYCLINE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 360 nm

Column: Xterra 150 x 4.6 mm, C8 column, with 3.5 μ m packing

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.5 mg/mL

Sample Solvent: Water

Mobile Phase: 0.1% v/v trifluoroacetic acid in water (pH 1.8) and acetonitrile (70:30)

Sample Injection Volume: 20 μ L

Flow Rate: 1.0 mL/min

Run Time: 5 minutes

Column Washing: Rinsed with 10 column volumes of 95:5 water-acetonitrile to remove traces of buffer salts, then 10:90 water-acetonitrile for 5 column volumes. Rinse with 5 column volumes of storage solution last.

Column Storage: 50:50 water-acetonitrile

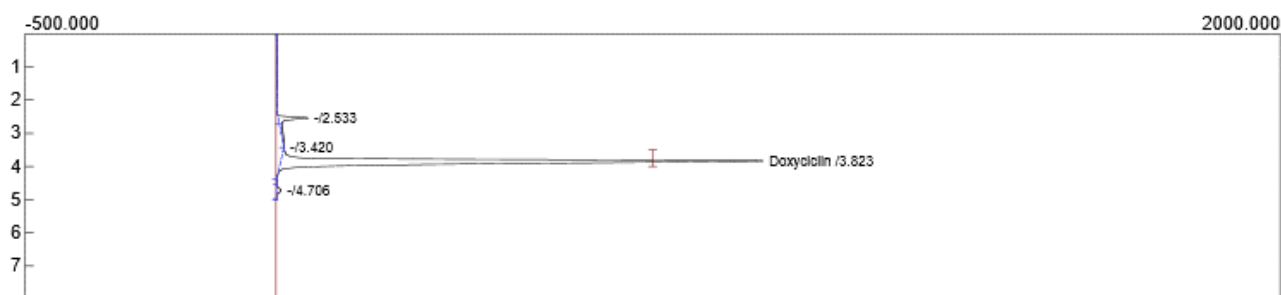
Analytical Metrics for Doxycycline

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Doxycycline Chromatogram



Instrument: Waters e2695 High Performance Liquid Chromatograph
Detector: Waters 2487 Dual-Wavelength Absorbance Detector
Analytical Wavelength: 208 nm
Column: Phenomenex 150 x 4.6 mm, C18 column, with 5.0 µm particle size
Column Temperature: Room temperature

Sample Concentration: 0.15 mg/mL
Sample Solvent: Buffer B and acetonitrile (95:5)
Buffer A: 2.8 g of monobasic sodium phosphate in 900 mL of water, adjust to pH 6.8 with 9 M sodium hydroxide solution, and dilute to 1000 mL with water
Buffer B: 2.8 g of monobasic sodium phosphate in 900 mL of water, adjust to pH 2.5 with phosphoric acid, and dilute to 1000 mL with water
Mobile Phase A: Acetonitrile
Mobile Phase B: Mixture of acetonitrile and Buffer A (5:95)
Sample Injection Volume: 30 µL
Flow Rate: 1.0 mL/min
Run Time: 15 minutes
Gradient Description:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Ramp
0	5	95	Equilibrating
2	5	95	Linear
4	25	75	None
5	60	40	None
8	60	40	Linear
9	25	75	None
10	5	95	None
15	5	95	Linear

Column Washing: Wash with 10 column volumes of 95:5 water-acetonitrile to remove any trace buffer salts and then wash with 10 column volumes of storage solution
Column Storage: 50:50 acetonitrile-water

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates
Tailing Factor USP protocol does not specify but should aim for <2.0
RSD for Replicate Injections <1.0%

Figure 1: A plot of the first 16 eigenvalues of the Laplacian operator on the domain Ω . The x-axis represents the eigenvalue, ranging from -500.000 to 2000.000. The y-axis represents the index of the eigenvalue, from 1 to 16. The plot shows two distinct groups of eigenvalues. The first group, labeled 'Manaslu 7.683', consists of eigenvalues 1 through 6, which are clustered near zero. The second group, labeled 'Enayapri 6.966', consists of eigenvalues 7 through 16, which are clustered around -1000.000. Each eigenvalue is marked with a vertical line segment and a horizontal line segment, indicating the range of the eigenvalue.

GENTAMICIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 330 nm

Column: Luna 100 x 4.6 mm, C18 column, with 5 µm packing

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.25 mg/mL

o-Phthalaldehyde Solution: Dissolve 1.0 g of o-phthalaldehyde in 5 mL methanol, and add 95 mL of 0.4 M boric acid, previously adjusted with 8 N potassium hydroxide to a pH of 10.4, and 2 mL thioglycolic acid. Adjust the resulting solution to a pH of 10.4.

Mobile Phase: Dissolve 5 g of sodium 1-heptanesulfonate in 700 mL methanol, 250 mL water, and 50 mL glacial acetic acid.

Sample Preparation: Prepare solution of gentamicin at 0.65 mg/mL in water. Transfer 10 mL of this solution and add 5 mL isopropyl alcohol and 4 mL o-Phthalaldehyde solution, mix, and add isopropyl alcohol to obtain 25 mL of solution. Heat at 60° in a water bath for 15 minutes, and cool.

Sample Injection Volume: 60 µL

Flow Rate: 1.5 mL/min

Run Time: 30 minutes

Column Washing: Rinsed with 10 column volumes of storage solution.

Column Storage: 50:50 water-methanol

Analytical Metrics for Gentamicin

Column Efficiency >1200 theoretical plates

Resolution between any two peaks <1.25

Capacity factor for the gentamicin C1 peak between 2-7

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.0%

Gentamicin C₁ (25-50%), C_{1a} (10-35%), sum of gentamicin C_{2a} and C₂ (25-55%)

Typical Gentamicin Chromatogram

LEVOFLOXACIN (Geneva College's Method Shown)

Instrument: Ultimate 3000 Dionex High Performance Liquid Chromatograph

Detector: RS VWD Ultimate 3000 Dionex Detector

Analytical Wavelength: 280 nm

Column: Luna LC 150 x 4.6 mm, C18 column, with 3.0 μ m particle size

Column Temperature: Room temperature

Gradient Method

Sample Concentration: 0.01 mg/mL

Sample Solvent: Acetonitrile and buffer (acetonitrile first)

Buffer: 6.16 g of ammonium acetate and 16.86 g of sodium perchlorate monohydrate in 2000 mL water, adjust to pH 2.2 with phosphoric acid

Mobile Phase A: Buffer and acetonitrile (84:16)

Mobile Phase B: Buffer and acetonitrile (50:50)

Sample Injection Volume: 20 μ L

Flow Rate: 1.0 mL/min

Run Time: 20 minutes

Gradient Description:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Ramp
0	100	0	Equilibrating
5	100	0	None
10	82	18	Linear
15	40	60	Linear
15.1	100	0	Linear
20	100	0	None

Column Washing: Wash with methanol for several column volumes before storage.

Column Storage: 80:20 water-methanol

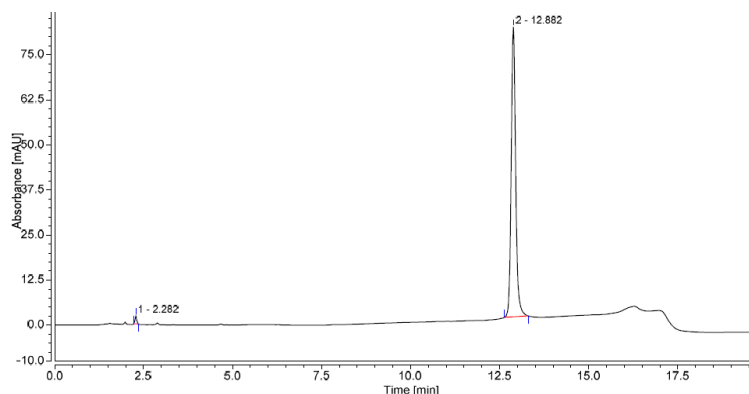
Analytical Metrics for Levofloxacin

Column Efficiency USP protocol does not specify but aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.0%

Typical Levofloxacin Chromatogram



LOSARTAN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 250 nm

Column: Symmetry 150 x 4.6 mm, C8 column, with 5 µm particle size

Column Temperature: Room temperature

Gradient Method

Sample Concentration: 0.25 mg/mL

Sample Solvent: Water

Buffer: 1.25 mg/mL of monobasic potassium phosphate and 1.5 mg/mL of dibasic sodium phosphate in water, pH is approximately 7.0

Mobile Phase A: Acetonitrile and Buffer (3:17)

Mobile Phase B: Acetonitrile

Sample Injection Volume: 30 µL

Flow Rate: 1.0 mL/min

Run Time: 10 minutes

Gradient Description:

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Ramp
0	80	20	Equilibrating
1	80	20	None
2	60	40	Linear
7	60	40	None
8	80	20	Linear
10	80	20	None

Column Washing: Wash with 5 column volumes of water to remove any trace buffer salts then wash with storage solution.

Column Storage: 50:50 acetonitrile-water.

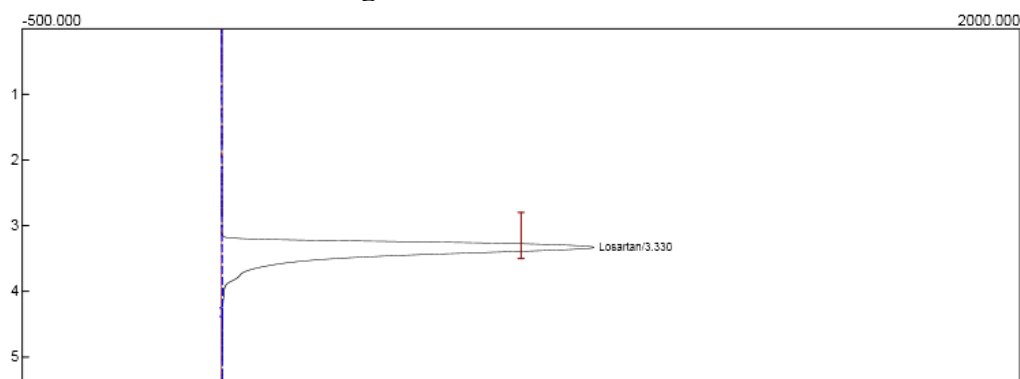
Analytical Metrics for Losartan

Column Efficiency >3000 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <2.0%

Typical Losartan Chromatogram



METFORMIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 218 nm

Column: XTerra 250 x 4.6 mm, C18 column, 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.5 mg/mL

Sample Solvent: Water

Buffer: 0.5 g/L of sodium octansulfonate and 0.5 g/L of NaCl in water brought to pH 3.85 with 0.06 M phosphoric acid

Mobile Phase: 20:80 Acetonitrile-Buffer

Sample Injection Volume: 10 µL

Flow Rate: 1.0 mL/min

Run Time: 12 minutes

Column Washing: 5 column volumes of 100% water to remove traces of buffer salts, then run 5 column volumes of storage solution.

Column Storage: 50:50 water-acetonitrile

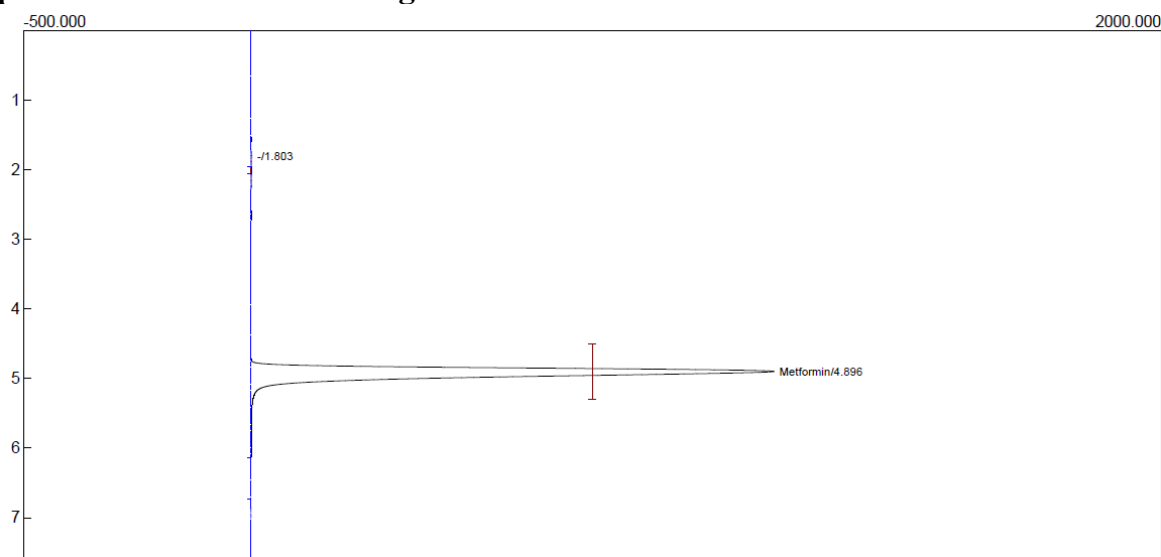
Analytical Metrics for Metformin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <2.0

RSD for Replicate Injections <1.5%

Typical Metformin Chromatogram



METHOTREXATE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 302 nm

Column: XBridge 250 x 4.6 mm, C18 column, 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.25 mg/mL

Sample Solvent: Mobile phase

Buffer: 0.2 M dibasic sodium phosphate and 0.1 M citric acid (630:370) adjust to pH 6

Mobile Phase: Acetonitrile and Buffer (10:90)

Sample Injection Volume: 20 µL

Flow Rate: 1.2 mL/min

Run Time: 8 minutes

Column Washing: Run 5 column volumes of 100% water to remove traces of buffer salts and then run 5 column volumes of 50% acetonitrile and 50% water.

Column Storage: 50:50 acetonitrile-water.

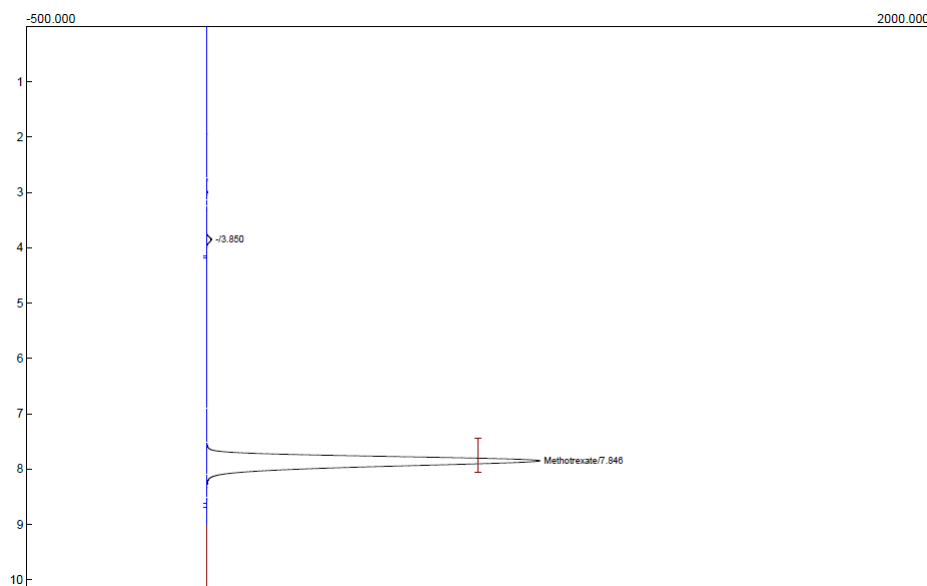
Analytical Metrics for Methotrexate

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.5%

Typical Methotrexate Chromatogram



OFLOXACIN (Indiana University- Bloomington's Method Shown)

Instrument: Agilent 1100 High Performance Liquid Chromatograph

Detector: Agilent 1100 Series

Analytical Wavelength: 330 nm

Column: Discovery 250 x 4.6 mm, C18 column, 5 μ m particle size

Column Temperature: 30 °C

Isocratic Method

Sample Concentration: 0.2 mg/mL

Sample Solvent: Methanol

Buffer: 1.25 g copper(II) sulfate pentahydrate and 1.31 g L-isoleucine in 1000 mL water

Mobile Phase: Methanol and Buffer (20:80)

Sample Injection Volume: 20 μ L

Flow Rate: 1.0 mL/min

Run Time: 15 minutes

Column Washing: Run 5 column volumes of 90:10 water-methanol and then run 5 column volumes of 50% methanol and 50% water.

Column Storage: 50:50 methanol-water.

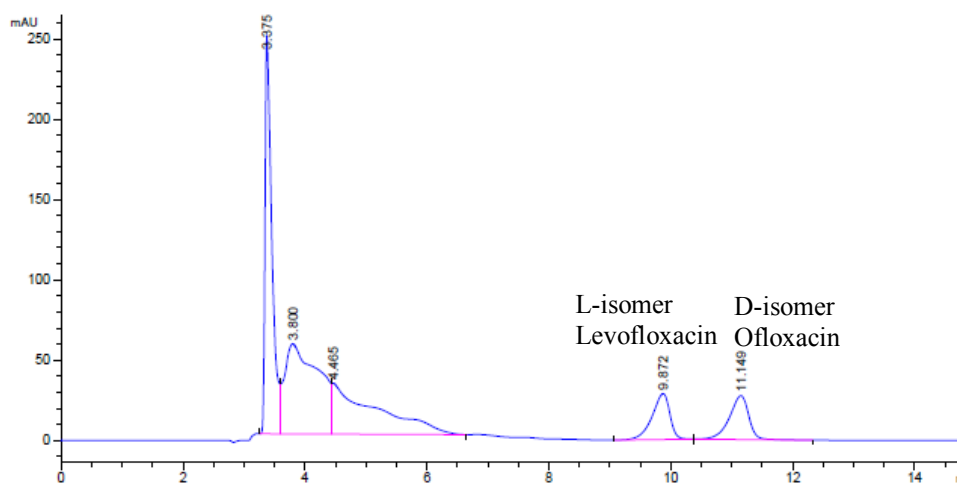
Analytical Metrics for Ofloxacin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.5%

Typical Ofloxacin Chromatogram



OMEPRAZOLE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 280 nm

Column: Xterra 150 x 4.6 mm, C8 column, 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.5 mg/mL

Sample Solvent: Acetonitrile and 0.01 M sodium borate (1:3)

Buffer: 0.725 g monobasic sodium phosphate, anhydrous and 4.472 g dibasic sodium phosphate, anhydrous in 1 L water. Dilute 250 mL of this solution with water to 1 L. Adjust with phosphoric acid to bring to pH 7.6. Store in a fridge when not in use.

Mobile Phase: Acetonitrile and Buffer (30:70)

Sample Injection Volume: 20 µL

Flow Rate: 1.2 mL/min

Run Time: 6 minutes

Column Washing: Run 5 column volumes of 100% water to remove traces of buffer salts and then run 5 column volumes of 50% acetonitrile and 50% water.

Column Storage: 50:50 acetonitrile-water.

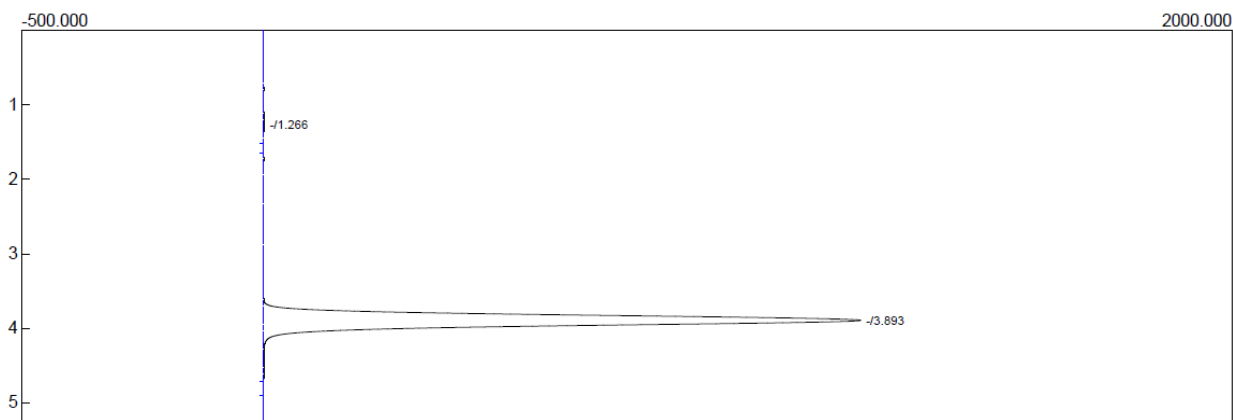
Analytical Metrics for Omeprazole

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <1.5

RSD for Replicate Injections <1.0%

Typical Omeprazole Chromatogram



OXALIPLATIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 210 nm

Column: XTerra MS 250 x 4.6 mm, C18 column, 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.4 mg/mL

Sample Solvent: 40 mg/mL lactose monohydrate solution

Buffer: 1.36 g monobasic potassium phosphate, anhydrous and 0.9 g 1-pentanesulfonic acid sodium salt in 1 L water. Add 250 µL triethylamine and about 5 drops of phosphoric acid to bring to pH 4.3. Store in a fridge when not in use.

Mobile Phase: Methanol and Buffer (15:85)

Sample Injection Volume: 40 µL

Flow Rate: 1.0 mL/min

Run Time: 8 minutes

Column Washing: Run 5 column volumes of 100% water to remove traces of buffer salts and then run 5 column volumes of 50% methanol and 50% water.

Column Storage: 50:50 methanol-water.

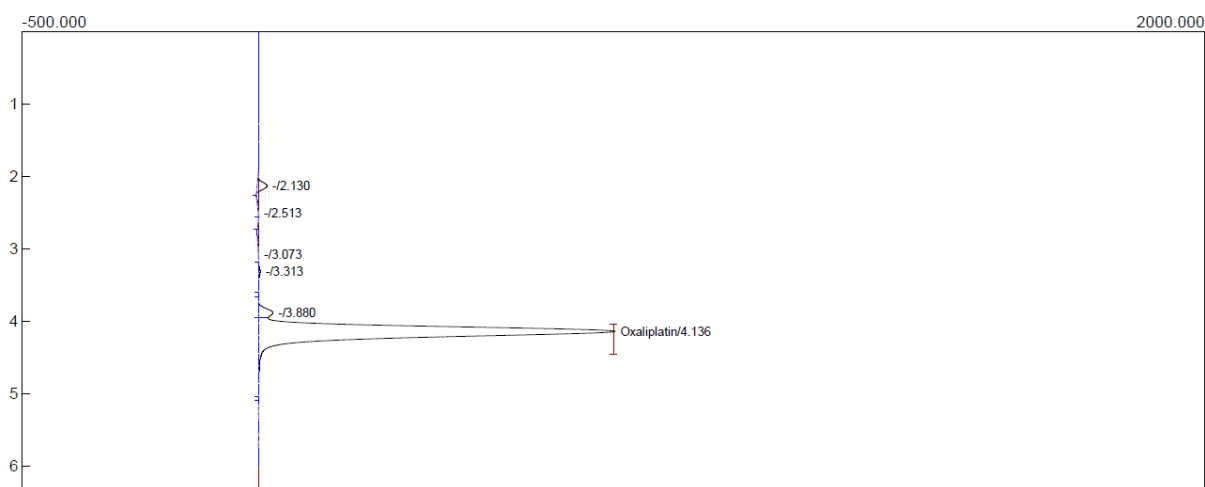
Analytical Metrics for Oxaliplatin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.5%

Typical Oxaliplatin Chromatogram



OXYTOCIN

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 220 nm

Column: Symmetry 150 x 4.6 mm, C18 column, with 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.5 mg/mL

Sample Solvent: Water

Mobile Phase A: Buffer solution of 0.1 M monobasic sodium phosphate

Mobile Phase B: 1:1 acetonitrile and water

Sample Injection Volume: 40 µL

Flow Rate: 1.5 mL/min

Run Time: 16 minutes

Isocratic: 30% Mobile Phase B and 70% Mobile Phase A.

Column Washing: 5 column volumes of 80:20 water-acetonitrile then 10 column volumes of storage solution.

Column Storage: 50:50 acetonitrile-water.

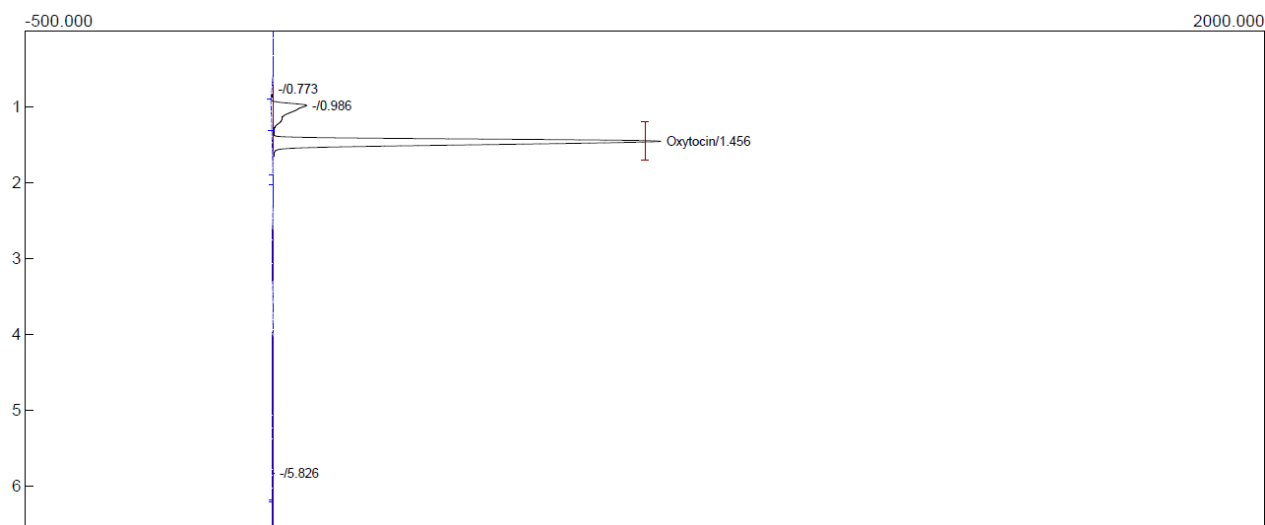
Analytical Metrics for Oxytocin

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <2.0

RSD for Replicate Injections <2.0%

Typical Oxytocin Chromatogram



PROPOFOL (USP Method Shown)

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 275 nm

Column: Symmetry 200 x 4.6 mm, Porous silica column, with 5 µm particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 2.4 mg/mL

Sample Solvent: Hexane

Mobile Phase: Hexane, acetonitrile, and alcohol (990:7.5:1)

Sample Injection Volume: 10 µL

Flow Rate: 2.0 mL/min

Run Time:

Column Washing:

Column Storage:

Analytical Metrics for Propofol

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <1.5

RSD for Replicate Injections <2.0%

Typical Propofol Chromatogram

QUININE

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 235 nm

Column: XTerra 250 x 4.6 mm, C18 column, with 5 μ m particle size

Column Temperature: Room temperature

Isocratic Method

Sample Concentration: 0.2 mg/mL

Sample Solvent: Mobile phase

Buffer A: 35.0 mL of methanesulfonic acid to 20.0 mL of glacial acetic acid, dilute with water to 500 mL

Buffer B: Dissolve 10.0 mL of diethylamine in water to obtain a 100 mL solution

Mobile Phase: Water, acetonitrile, Buffer A, and Buffer B (860:100:20:20). Adjust with Buffer B to a pH of 2.6 if found to be lower.

Isocratic Mobile Phase: Mobile Phase and Acetonitrile (92:8)

Sample Injection Volume: 50 μ L

Flow Rate: 1.0 mL/min

Run Time: 10 minutes

Column Washing: 10 column volumes of water to remove trace buffer salts and then 5 column volumes of storage solution

Column Storage: 50:50 acetonitrile-water

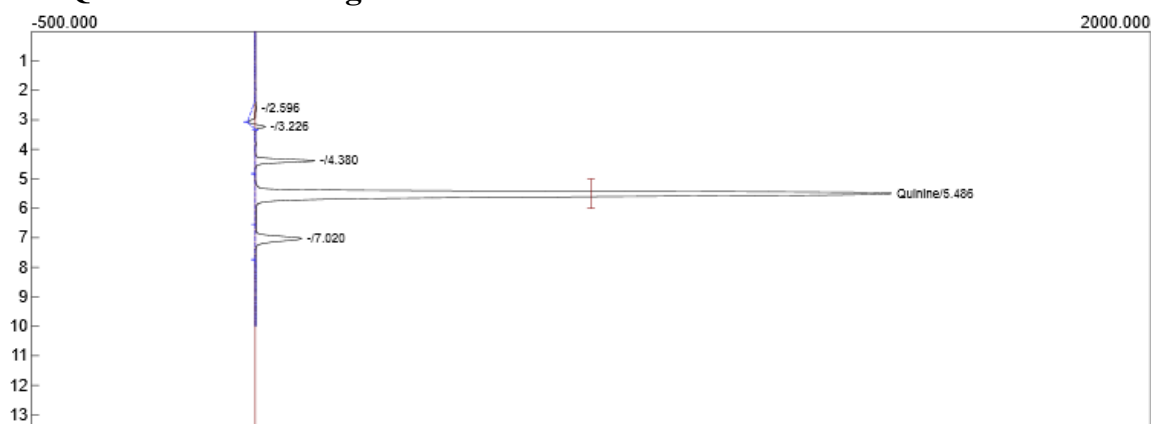
Analytical Metrics for Quinine

Column Efficiency USP protocol does not specify but aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <1.5

RSD for Replicate Injections <2.0%

Typical Quinine Chromatogram



SILDENAFIL (USP Method Shown)

Instrument: Waters e2695 High Performance Liquid Chromatograph

Detector: Waters 2487 Dual-Wavelength Absorbance Detector

Analytical Wavelength: 290 nm

Column: Brand 150 x 3.9 mm, C18 column, with 5 µm particle size

Column Temperature: 30 °C

Isocratic Method

Sample Concentration: 0.028 mg/mL

Sample Solvent: Mobile phase

Buffer: 7 mL of triethylamine diluted to 1000 mL with water, adjust to pH 3.0 with phosphoric acid

Mobile Phase: Buffer, methanol, and acetonitrile (58:25:17)

Sample Injection Volume: 20 µL

Flow Rate: 1.0 mL/min

Run Time:

Column Washing:

Column Storage:

Analytical Metrics for Sildenafil

Column Efficiency USP protocol does not specify but should aim for >1500 theoretical plates

Tailing Factor <1.5

RSD for Replicate Injections <0.85%

Typical Sildenafil Chromatogram

SULFAMETHOXAZOLE-TRIMETHOPRIM (Newman University's Method Shown)

Instrument: Agilent 1260 Infinity II LC

Detector: 1260 Infinity II Diode Array Detector HS

Analytical Wavelength: 254 nm

Column: InfinityLab Poroshell 120 100 x 4.6 mm, C18 column, with 2.7 μ m particle size

Column Temperature: 45 °C

Isocratic Method

Sample Concentration: 0.16 mg/mL sulfamethoxazole and 0.032 mg/mL trimethoprim

Sample Solvent: Mobile phase

Mobile Phase: 700 mL water, 200 mL acetonitrile, and 1 mL triethylamine in a 1000 mL volumetric flask. Allow to equilibrate at room temperature, and adjust with 0.2 M sodium hydroxide or dilute glacial acetic acid (1/100) to pH 5.9 \pm 0.1. Bring to volume with water.

Sample Injection Volume: 20 μ L

Flow Rate: 2 mL/min

Run Time: 3 minutes

Column Washing: Rinse with water for 10 column volumes before bringing to storage conditions.

Column Storage: 50:50 methanol-water

Analytical Metrics for Sulfamethoxazole-Trimethoprim

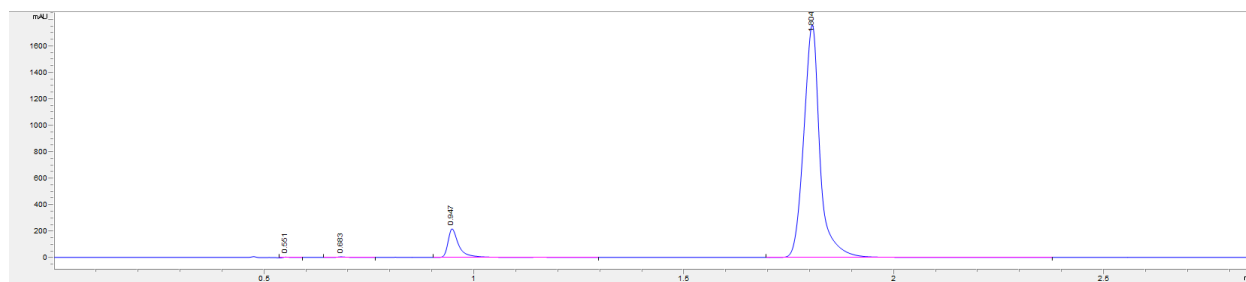
Resolution between the sulfamethoxazole and trimethoprim peaks >5.0

Column Efficiency USP protocol does not specify but aim for >1500 theoretical plates

Tailing Factor USP protocol does not specify but should aim for <1.5

RSD for Replicate Injections <2.0% for both APIs

Typical Sulfamethoxazole-Trimethoprim Chromatogram



REVISION HISTORY

This manual was drafted 2014-11-04

Updated 2015-01-28 to fix errors in amoxicillin/amoxy-clav gradients Updated 2015-04-16 to fix more gradient errors.

Updated 2015-05-15 to add QA/QC procedures

Updated 2015-05-19 to add information on column storage, washing, reconditioning

Initials/Date	Change	Purpose
NM 2015-08-07	Deleted Ampicillin analysis from the Amp, Amox, Amox/Clav analysis	The parameters are only valid for Amox and Amox/Clav. The analyses have been separated to shorten run time. A new section will be added for Ampicillin analysis.
NM 2015-08-07	Gradient for Amox and Amox/Clav has been updated	Run time is shorter
ML 2015-08-07	Corrected cipro buffer to include 3.5% acetonitrile	Buffer description was incorrect
ML 2015-08-20	Added a form to summarize method	
NM & ML 2015-11-06	Rewrote some confusing text	Clarify verification requirements
ML 2016-03-25	Added cipro sample prep directions	
ML & NM 2016-05-12	Made validation form more explicit/transparent	
MB 2016-06-28	Formatting changes	Clarification and use with electronic OSF templates for information submission
MB 2016-07-14	Added to Legal Considerations	OSF security considerations
ML & SB 2017-07	Added additional USP methods	
SB 2018-07-30	Added to Methods	Updated the section for methods validated by ND
SB 2019-02-19	Added to Methods	Updated the section for new methods validated by ND and DPAL participant schools
SB 2019-09-27	Added to Methods	Updated for new methods validated by ND and DPAL participant schools
SB 2020-03-05	Updated Methods and clarified System Suitability Requirements	Updated new methods validated at ND and clarified material used for system suitability tests