Appendix

Record to standardize sample preparation procedures in a glycoanalytical SOP

To minimize mistakes in the procedures described above and to improve the standardisation of the sample preparation, a protocol was created.

Pre-calculations:

Sample	Protein (mg)	Volume of incubation buffer	1. trypsin addition	2. trypsin addition	Total trypsin	1. PNGaseF	2. PNG-ase F	Total PNGaseF
1						25 µl	25 µl	50 mU
2						25 µl	25 µl	50 mU
3						25 µl	25 µl	50 mU
4						25 µl	25 µl	50 mU
5						25 µl	25 µl	50 mU

Desalting:

Small quadrates in the table should be marked after performing the specific step.

Sample	Adjusting volumes of samples to 2.5 ml	Flushing of the PD-10-columns with 25 ml purified water:	Pipetting of samples	Elution of the samples with 3.5 ml purified water	Lyophilization
1	□	□	□	□	□
2	□	□	□	□	□
3	□	□	□	□	□
4	□	□	□	□	□
5	□	□	□	□	□

Trypsin-digestion:

Sample	Dissolving in incubation buffer	1. trypsin addition	2. trypsin addition	Cooking the samples for 10 minutes
1	□	□	□	□
2	□	□	□	□
3	□	□	□	□
4	□	□	□	□
5	□	□	□	□

PNGaseF-digestion:

Sample	1. PNGaseF addition	2. PNGaseF addition
1	◻	◻
2	◻	◻
3	◻	◻
4	◻	◻
5	◻	◻

Purification by cation exchange resins:

Sample	Addition of 100 µl 10% acetic acid	Flushing of the disposable columns with 1 ml purified water	Filling in 0.5 ml cation exchange resin	Flushing with 5*0.5 ml purified water	Flushing with 5*0.5 ml 1% acetic acid
1	◻	◻	◻	◻ ◻ ◻ ◻ ◻	◻ ◻ ◻ ◻ ◻
2	◻	◻	◻	◻ ◻ ◻ ◻ ◻	◻ ◻ ◻ ◻ ◻
3	◻	◻	◻	◻ ◻ ◻ ◻ ◻	◻ ◻ ◻ ◻ ◻
4	◻	◻	◻	◻ ◻ ◻ ◻ ◻	◻ ◻ ◻ ◻ ◻
5	◻	◻	◻	◻ ◻ ◻ ◻ ◻	◻ ◻ ◻ ◻ ◻

Sample	Transfer of the sample onto the cation exchange resin	Flushing with 4*0.5 ml purified water	Lyophilization	Transfer of the samples in PCR-tubes	Concentration of the samples to a volume of 10 µl (100µl, 50µl, 20µl, 10µl)
1	□ □ □ □	□ □ □ □	□	□ □ □ □	□ □ □ □
2	□ □ □ □	□ □ □ □	□	□ □ □ □	□ □ □ □
3	□ □ □ □	□ □ □ □	□	□ □ □ □	□ □ □ □
4	□ □ □ □	□ □ □ □	□	□ □ □ □	□ □ □ □
5	□ □ □ □	□ □ □ □	□	□ □ □ □	□ □ □ □

2-AB-Labelling:

Sample	Addition of 10 µl labelling-mixture and Incubation for 16 h
1	□
2	□
3	□
4	□
5	□

Purification of the labelled oligosaccharides:

a – samples:

Sample	1. Paper-chromatography	1. Drying of the paper stripes	2. Paper-chromatography	2. Drying of the paper stripes	Cutting off sample spot
1.a	□	□	□	□	□
2.a	□	□	□	□	□
3.a	□	□	□	□	□
4.a	□	□	□	□	□
5.a	□	□	□	□	□

Sample	Elution with 3*1 ml purified water	Lyophilization	Transfer into 0.5 ml screwing vials	Concentration of the samples to 100 µl
1.a	□ □ □	□	□ □ □ □	□
2.a	□ □ □	□	□ □ □ □	□
3.a	□ □ □	□	□ □ □ □	□
4.a	□ □ □	□	□ □ □ □	□
5.a	□ □ □	□	□ □ □ □	□

b – samples:

Sample	1. Paper-chromatography	1. Drying of the paper stripes	2. Paper-chromatography	2. Drying of the paper stripes	Cutting off sample spot
1.b	□	□	□	□	□
2.b	□	□	□	□	□
3.b	□	□	□	□	□
4.b	□	□	□	□	□
5.b	□	□	□	□	□

Sample	Elution with 3*1 ml purified water	Lyophilization	Transfer into 0.5 ml screwing vials	Concentration of the samples to 30 µl
1.b	□ □ □	□	□ □ □ □	□
2.b	□ □ □	□	□ □ □ □	□
3.b	□ □ □	□	□ □ □ □	□
4.b	□ □ □	□	□ □ □ □	□
5.b	□ □ □	□	□ □ □ □	□

Sialidase digestion:

Sample	Addition of 10 µl sialidase buffer	Addition of 10 µl sialidase and incubation for 48 h
1.b	□	□
2.b	□	□
3.b	□	□
4.b	□	□
5.b	□	□

Mixed bed purification:

Sample	Flushing of the disposable columns with 1 ml purified water	Filling in 0.5 ml cation exchange resin	Filling in 0.5 ml anion exchange resin	Flushing of the mixed bed with 5*2 ml purified water	Transfer of the b-samples onto the mixed bed
1.b	□	□	□	□ □ □ □ □	□ □ □ □
2.b	□	□	□	□ □ □ □ □	□ □ □ □
3.b	□	□	□	□ □ □ □ □	□ □ □ □
4.b	□	□	□	□ □ □ □ □	□ □ □ □
5.b	□	□	□	□ □ □ □ □	□ □ □ □

Sample	Flushing the mixed bed (1*0.5 ml, 1*1 ml, 2*2 ml)	Lyophilization	Transfer of the sample into 0.5 ml Screwing vials	Concentration to 100 µl
1.b	□ □ □ □	□	□ □ □ □	□
2.b	□ □ □ □	□	□ □ □ □	□
3.b	□ □ □ □	□	□ □ □ □	□
4.b	□ □ □ □	□	□ □ □ □	□
5.b	□ □ □ □	□	□ □ □ □	□

Qualification of the HPLC-device

As mentioned above, ProBioGen produced biological API according to GMP-guidelines. The manufacturing facility had a Quality Management System according to ISO-DIN 9001:2000 and was certified according to EU-guidelines for the manufacturing of investigational medicinal products.

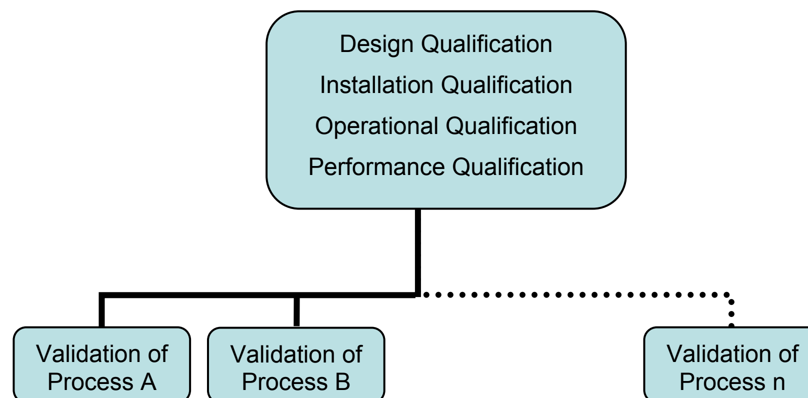
Due to this ISO-certification, all process critical devices at ProBioGen were qualified. In general, the qualification process consists of four steps:

- Design Qualification (DQ)
- Installation Qualification (IQ)
- Operational Qualification (OQ)
- Performance Qualification (PQ)

To establish the described glycoanalytical methods at ProBioGen, a new HPLC-device had to be aquired. This new HPLC-device had been qualified before the validation of the HPLC-methods started.

Background

Technical systems for validation-obligatory processes for the production of APIs and drugs have to be qualified according to Annex 15 of the EG-GMP-guideline and the PIC/S PI006-2. The qualification of technical systems as basis for the validation of processes takes place in different stages:



Each qualification step consists of a qualification plan, protocols and a report. The Performance Qualification and the following process validation are often not clearly circumscribable and pass over into each other.

Risk analysis

The risk analysis is an important tool to judge deficiencies of devices and methods. The documented risk analysis is therefore a record for external auditors that devices and procedures are fully understood and manageable.

It is best to start the risk analysis before the device is acquired to judge the device and its environment in the laboratory. Thereby arrangements for minimizing the risks are appointed.

The influence of every analysis- or production-step with influence on the product quality is theoretically judged. Every analysis- or production-step with influence on the product quality is judged as crucial and must be evaluated during qualification. The protocols consist of process descriptions and corresponding specifications. Every analysis- or production-step with no influence on product quality is judged as non-crucial and must not be

evaluated in the qualification procedure.

There are different methods for risk analysis available. In this work, the Failure Mode and Effects Analysis (FMEA) was chosen for device qualification purposes and the method by Ishikawa was used for method validation aspects [165].

Within FMEA three parameters are evaluated:

- Failure frequency (F)
- Severity code (S)
- Likelihood of failure detection (L)

Each parameter is evaluated by a number from 1 to 5, although this is arbitrary. Thereby it is tried to list up all important device functions and all imaginable failures by experience.

Judgement criteria	1	2	3	4	5
Failure frequency (F)	low				high
Severity code (S)	low				high
Likelihood of failure detection (L)	high				low

The assigned points for each parameter are multiplied and the result is a risk factor which can be between 1 and 125. The risk factor is a degree for the relative risk. In general, a device function should be evaluated during qualification whether the risk factor is higher than 25-30.

In this case, the Design Qualification was done before the Risk analysis. Its major goal was to find the crucial parameters for the following IQ, OQ and PQ. The risk factor was set at 30.

Design Qualification

The aim of the DQ is the evaluation, in how far the product requirement specifications comply with the customer requirement specifications. The customer requirement specifications are created by the customer and represent a list of requirements for a certain device. The product requirement specifications are a technical solution proposal of a manufacturer for the customer requirements.

Customer requirement specifications for a HPLC-device

The HPLC-device should be able to do the following applications:

- Peptide Mapping for protein identification
- Purity Map for the determination of impurities
- HILIC as method for glycosylation analysis of uncharged glycans
- AEX as method for glycosylation analysis of charged glycans
- Preparative protein purification by a low pressure gradient

Therefore the following device components were necessary:

- 2 HPLC-pumps for a high pressure gradient system, necessary for the analytical applications
- Low pressure control valve for the preparative application
- Degasser, for degassing the eluents
- UV-detector for protein and peptide analysis
- Fluorescence-detector for glycosylation analysis
- Autosampler for high-throughput-analysis
- Fraction collector for preparative applications
- Column oven to improve repeatability of chromatograms

In addition, the HPLC-device should meet the following requirements:

- Manual and computer-assisted control

- Network-capability
- Equipment reliability
- Good Service
- Certified Manufacturer

<u>Product requirement specifications</u>

<u>Device acquirement:</u>

After a pre-selection of HPLC-manufacturers, the following companies were considered:

- VWR International GmbH
- Shimadzu Deutschland GmbH
- Waters GmbH

In the following price-competition Shimadzu Deutschland GmbH could prevail.

<u>Shimadzu product requirement specifications:</u>

<u>HPLC Pump: LC-10ATvp</u>

- ability for high- and low-pressure gradient
- Flow rate setting range: 0.001 - 5 ml/min [1.0 - 39.2 MPa]
- Flow rate setting range: 5.001 - 9.999 ml/min [1.0 - 19.6 MPa]
- robust double reciprocating plunger pump for routine analysis
- Flow rate accuracy: ± 2%
- Flow rate precision: ± 0.3%
- Pressure setting range: 1.0 - 39.2 MPa in 0.1 MPa steps
- Pressure accuracy: ± 10%
- Upper and lower pressure limits
- Liquid contacting part materials: SUS316, ruby, sapphire, Teflon®, HastelloyC®, PEEK, PCTFE, zirconia, perfluoroelastomer

- Suction filter: 10 µm mesh
- Line filter: 5 µm mesh
- Time program: Commands for flow rate, pressure, EVENT functions, LOOP function, 10 files, total 320 steps
- Pressure display accuracy: ± 2%

UV-VIS Detector: SPD-10Avp

- Light source: Deuterium lamp
- Wavelength range: 190 - 600 nm
- Spectral bandwidth: 8 nm
- Wavelength accuracy: ± 1nm
- Wavelength reproducibility: ± 0.1 nm
- Drift: 2×10^{-4} AU/hour Max. (250 nm, room temperature constant, air in the cell)
- Noise level: ± 0.35 x 10-^{5} AU Max. (250 nm, air in the cell)
- Cell path length: 10 mm
- Cell volume: 8 µl
- Cell pressure tolerance 12 MPa
- Cell materials in contact with liquid: SUS316, Teflon®, quartz
- Dual wavelength mode: 190 - 370 nm or 371 - 600 nm range
- Sampling frequency: 1.2 sec for one wavelength
- Response: Selectable in 10 steps corresponding to time constant 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 6.0. 8.0 and 10.0 secons
- Range: Can be set between 0.0001 and 2.56 AUFS in 0.0001 AUFS steps

Fluorescence Detector: RF-10Axl

- Light source: 150 W xenon lamp
- Light source chamber: Ozone self dissolving type lamp house
- Monochromators: Ion brazed holographic concave diffraction grating monochromators for both excitation and emission sides
- Number of grooves: 900 lines/mm
- Wavelength range: 200 - 650 nm
- Bandwidth: 15 nm for both excitation and emission sides
- Wavelength accuracy: ± 2 nm
- Wavelength repeatability: ± 0.2 nm
- Wavelength scanning speed: from 24 nm/min - 3,000 nm/min
- Wavelength moving speed: 15.000 nm/min
- Detector: Signal Side: Photomultiplier tube, Monitor side: Photodiode
- Measuring method: Ratio compensation system with monochromatic light monitoring
- Time constant: 0.1, 0.5, 1.5 and 3 s
- Sensitivity: HIGH, MED and LOW (sensitivity difference is approx. 32 times)
- Gain: x1, x4 and x16
- Range: 1/∞ (0 mV), x1, x1/2, x1/4, x1/8, x1/16, x1/32, x1/64, x1/128, x1/256
- Cell: 12 µl square quarts flow cell, pressure tolerance 1.96 MPa
- S/N: at the Raman peak of distilled water is 300 or higher (when EX = 350 nm and Response = 1.5 s

Column oven: CTO-10ASvp

- Block heating type
- Temperature control range: (Room temperature - 15^oC) - (Room temperature + 60^oC)

- Temperature setting range: 4 - 80°C in 1°C steps
- Temperature control precision: ± 0.1^{o}C
- Ambient temperature range: 4 - 35°C
- Size of columns housed: 250 mm columns
- Preheater capacity: 10 µl x 2 flow lines

Low-Pressure Gradient Flow Control Valve: FCV-10ALvp

- 4 channels
- Cooling of the control valves
- Fast valve times
- Minimal dead volume

On-line Degasser: DGU-14A

- 4 channel online membrane degasser
- Max. flow rate 20 ml/min per channel

Auto Injector: SIL-10ADvp

- Variable injection volume type (no sample loss injection)
- Injection volume setting range: 0.1 - 50 µl in 0.1 µl increments
- Sample processing capacity: 70 vials à 2.2 ml
- Number per repeat injections: 1 to 30 per sample
- Run time: Can be set between 0.01 and 9999.9 minutes in 0.01 minute increments
- Sample aspiration speed: variable (between 0.1 - 15 µl/sec)
- Rinse solvent aspiration rate: variable (between 1 and 35 µl/sec)
- Injection reproducibility: C.V. 0.3% or less for 10 µl injection
- Injection accuracy: Injection volume compensation possible
- Operating pressure: Max. 34.3 MPa

- Operating pH: pH 1 - 10

Fraction collector: FRC-10A

- Driving system: Moving arm [X-Y] system
- Number of ports: 144
- Fractionating system: Fractionation with solenoid valve
- Maximum flow rate: 150 ml/min (water)
- Automatic fractionation by UV-VIS- and RF-Peak-signals

System Controller: SCL-10Avp

- Central control module for all modules of the vp-series
- Data transfer to the PC via optical link
- Validation functions for the whole system
- Self tests possible

Personal Computer: Fujitsu-Siemens C2400-W2000

- Processor: Intel P4, 2.4 GHz, 400 MHz FSB
- Hard disk: 40 Gb
- Dongle for CLASS-VP-Software

Software: Shimadzu CLASS-VP

- Version 6.12 Service Pack 5

Risk assessment for the qualification procedures

During the risk assessment for qualification procedures each working step is evaluated regarding its influence on the quality of the result. For evaluation the FMEA-method was used.

	Considerations					**After sanction**			
Risk	**F**	**S**	**L**	**Risk factor (FxSxL)**	**Sanction**	**F**	**S**	**L**	**Risk factor (FxSxL)**
Flow rate accuracy	2	5	3	30	Annual servicing of the plunges and the line filters; Control in OQ ("Flow test") and in the requalification process	1	5	3	15
Flow rate precision (Flow Pulsation)	2	5	3	30	Annual servicing of the plunges and the line filters; Control in OQ ("Flow pulsation test") and in the requalification process	1	5	3	15

Gradient-precision - High pressure-gradient - Low pressure-gradient	3	5	3	45	Annual servicing of the plunges and the line filters; Control in OQ ("Gradient system test") and in the requalification process	1	5	3	15
Closeness	4	3	2	32	Control in OQ and in the requalification process by visual inspection and leakage control	2	3	1	6
Back plunger rinsing dry	5	2	2	20	Visual control; Change of the 20% degassed ethanole one time per month and within the requalification process	2	2	2	8

Wavelength accuracy of both detectors	2	5	5	50	Control in OQ (UV: "Wavelength Accuracy“ RF: "Wavelength Accuracy Emission and Excitation“) and in the Performance Test	1	5	3	15
Base line noise of both detectors	4	4	2	32	UV-detector: Control in OQ ("Lamp Energy Test“) and in the requalification process RF-detector: Control in OQ ("Sensitivity and S/N Test“) and in the requalification process	2	4	2	16

Base line drift of both detectors	4	4	2	32	Determination of the base line drift takes place within the particular method validation	4	4	1	16
Linearity of both detectors	2	5	5	50	Determination of the base line drift takes place within the particular method validation	2	5	2	20
Lamp intensities of both detectors	2	5	3	30	Control of UV-lamp intensity in the OQ ("Lamp Energy Test") and in the requalification process Control of lamp intensity of RF-detector in the OQ (Determination of the	1	5	2	10

					Raman-Peaks) and in the requalification process				
Vial position recognition of SIL and FRC	2	5	5	50	Control in the OQ ("Position Sensor Test“ of SIL and „Manual Collection Test“ of FRC) and in the requalification process	2	5	2	20
Injection precision of SIL	3	5	3	45	Control in the OQ („Injection Volume Repeatability Test“) and in the requalification process	1	5	3	15
Procrastination of SIL	3	5	4	60	Control in the PQ and in the requalification process	1	5	3	15

Temperature accuracy of the column oven	2	5	3	30	Control in the OQ („Oven heating and temperature stability test“) and in the requalification process	1	5	2	10
Temperature-precision of the column oven	2	5	3	30	Control in the OQ („Oven heating and temperature stability test“) and in the requalification process	1	5	2	10
Functional efficiency of the system controller and the PC	2	5	3	30	Control during OQ („System communication test“) and in the requalification process	1	5	2	10
Check of the alarm function	2	5	3	30	Check during alarm setting; Testing also during OQ and in the requalification process	2	5	1	10

To minimize the risk as much as possible, operators had to be trained and regular checks, calibrations, purifications and services were to be performed. A logbook had to be created for all relevant documentation.

Installation Qualification

The IQ made sure that all acquired device components were intact and completed and listed up all components with their serial numbers. Besides all delivered documentation for the device had to be checked and a device logbook had to be prepared.

A short summary of the IQ is shown below:

Manufacturer: Shimadzu

Device type: High Performance Liquid Chromatography System

Device name: QC-HPLC

Serial number: ODN-03-0010-22

Inventory number: 691188/1-10

Location: ProBioGen Room 109

Responsibility: Stefan Zietze

Date of delivery: 21.11.2003

Initial operation: 27.11.2003

Nominal	Actual	Inventory number/ Serial number	Evaluation
HPLC	HPLC	-/ ODN-03-0010-22	correct
System Controller	SCL-10AVP	691188/ C21014112698	correct
Pump 1	LC-10ATVP(B)	691188/1 C20974133246	correct

Pump 2	LC-10ATVP(A)	691188/2/ C20974133249	correct
Low-Pressure Gradient Flow Control Valve	FCV-10ALVP	691188/3/ C21084104260	correct
Degasser	DGU-14A	691188/4/ SDG 31362	correct
UV-VIS-Detector	SPD-10AVP	691188/5/ C20994133126	correct
Fraction Collector	FRC-10A	691188/6/ C20374103359	correct
Auto Injector	SIL-10ADVP	691188/7/ C21054008643	correct
Column Oven	CTO-10ASVP	691188/8/ C21044133088	correct
Fluorescence Detector	RF-10AXL	691188/9/ C20954103836	correct
Personal Computer	Fujitsu Siemens	691188/10/ YBEM 478301	correct
Monitor	17''-TFT-Display Fujitsu 43B1	-	correct

Keyboard	PS/2 Win95	-	correct
Mouse	PS/2, Standard	-	correct
User Manuals	User's Manual Spectrofluometric Detector RF-10AxL User's Manual Solvent Delivery Module LC-10ATVP (Instruction Manual) User's Manual UV-VIS Detector SPD-10AVVP (Instruction Manual) User's Manual Fraction Collector Module FRC-10A User's Manual Sytem Controller SCL-10AVP (Instruction Manual) User's Manual Auto Injector SIL-10ADVP (Instruction Manual) User's Manual Fraction Collector FRC-10A User's Manual Column Oven CTO-10ASVP (Instruction Manual) User's Manual LC Workstation Class-VP	-	correct

	(Quick Manual) User's Manual LC Workstation Class-VP (Instruction Manual) User's Manual Drain tube kit for Shimadzu Instruction Manual On-line Degasser DGU-14A Instruction Manual FCV-10ALVP- Low Pressure Gradient Flow control valve		
Manufacturer-Certificates	Certificates of all device components were available	-	correct
Further documents	Service contract	-	correct

Operational Qualification

In the Operational Qualification all functions, error messages and the programmability of the device were tested and a calibration was performed. The manufacturer Shimadzu Deutschland GmbH had some automated qualification procedures built in its device components. It would go beyond the scope of this work to describe them all in detail. Only acceptance criteria and parameter values for verification are showed.

Test Name	Acceptance criteria	Parameter values for verification	Result
SCL-10AVP:			
Floppy disk drive test	The procedure of disk formatting, file saving and file loading should work properly.	N/A	correct
Diagnostic test for ROM and RAM, LCD and Keyboard	No error message is displayed after the test.	Keyboard Test: passed Display Test: passed RAM/ROM Test: passed	correct
Firmware Version Verification	Version 5.42	Version 5.42	correct
Display Contrast Adjustment Test	A successful test result is shown according to the Method.	N/A	correct
Menu Screen Verification	All screens displayed are the same as the screens expected.	Sequence 0: passed Analysis File: passed Edit 2: passed Monitor 3: passed	correct

		System 4: passed Maintenance 5: passed	
System Control	Programmed sequence is successfully executed.	Auto Injector: passed A Pump: passed B Pump: passed Detector: passed	correct
Memory Backup	All parameters entered are retained in memory.	N/A	correct
FCV-10ALVP:			
Operational Testing	LEAK LED should lit for a moment	Leak LED lits: passed	correct
Solenoid Valve Test	Correct valve switching	Port A (LED & Flow): passed Port B (LED & Flow): passed Port C (LED & Flow): passed Port D (LED & Flow): passed	correct
DGU-14A:			
Normal Operation Test	Monitor LEDs illuminates as expected	Power (LED): passed Control (LED): passed Leak (LED): passed	correct

LC-10ATVP: Pump A			
Diagnostic Test for ROM and RAM	Memory Check RAM OK ROM OK should be displayed.	Memory Check: passed	correct
Firmware Version Verification	Version number is 5.28	Version 5.28	correct
Display and LED Test	A successful test result is shown according to the Method.	LED Function: passed Display Function: passed	correct
Time Program Test	A successful test result is shown according to the Method.	Time Program: passed	correct
Flow Pulsation Test	Standard: Pulsation < 5 kg/cm^2	Pulsation: passed	correct
Pressure Limiter Test	MIN and MAX errors are activated.	MIN: passed MAX: passed	correct
Factory Setting Verification	All parameters listed in the PARAMETER VALUES section are displayed.	ALPHA: 4.3 PRS-5: 4770 T2: 10311 T4: 400 T1: 980 P-HP: 2560	correct

		T3: 101	
Flow Test	Measured Flow = 1.50 ± 0.05 ml/min	Flow 1: 1.508 ml/min Flow 2: 1.500 ml/min Flow 3: 1.500 ml/min Flow 4: 1.497 ml/min Flow 5: 1.497 ml/min Actual Flow: 1.501 ml/min	correct
<u>LC-10ATVP: Pump B</u>			
Diagnostic Test for ROM and RAM	Memory Check RAM OK ROM OK should be displayed.	Memory Check: passed	correct
Firmware Version Verification	Version number is 5.28	Version 5.28	correct
Display and LED Test	A successful test result is shown according to the Method.	LED Function: passed Display Function: passed	correct
Time Program Test	A successful test result is shown according to the Method.	Time Program: passed	correct
Flow Pulsation Test	Standard: Pulsation < 5 kg/cm^2	Pulsation: passed	correct

Pressure Limiter Test	MIN and MAX errors are activated.	MIN: passed MAX: passed	correct
Factory Setting Verification	All parameters listed in the PARAMETER VALUES section are displayed.	ALPHA: 4.3 PRS-5: 4854 T2: 10311 T4: 400 T1: 980 P-HP: 2560 T3: 101	correct
Flow Test	Measured Flow = 1.50 ± 0.05 ml/min	Flow 1: 1.504 ml/min Flow 2: 1.497 ml/min Flow 3: 1.489 ml/min Flow 4: 1.524 ml/min Flow 5: 1.489 ml/min Actual Flow: 1.5006 ml/min	correct
SIL-10ADVP:			
Initialization Test	All LEDs should lit for a moment, each dot of each display matrix should be highlighted for a moment and no error message should be displayed at the end of initialization.	LED Function: passed Display Function: passed No Error Message displayed: passed	correct

Diagnostic Test for ROM and RAM	Memory Check RAM OK ROM OK should be displayed.	Memory Check: passed	correct
Position Sensor Test	POSITION SENSOR SENSOR GOOD should be displayed.	Position Sensor: passed	correct
Leak Sensor Test	The message: LEAK SENSOR TEST SENSOR GOOD is displayed.	Leak Sensor Check: passed	correct
Injection Volume Repeatability Test	%RSD (of peak area) < 0.5%	%RSD = 0.376%	correct
FRC-10A:			
Power Supply LED Test	The LED in front door of FRC-10A is lit.	N/A	correct
Initialization Test	A successful test result is shown according to the Method.	Initialization: passed	correct
Manual collection Test	The fraction arm should move according the key operation.	Manual Collection: passed	correct

Fractionation Test	One peak of an anthracene/pyrene-mixture should be fractionated. The fraction should only consist of mobile phase and one component.	Automatic Collection: passed	correct
CTO-10ASVP:			
Firmware Version Verification	Version: 5.25	Version: 5.25	correct
Diagnostic Test for ROM and RAM	Memory Check RAM OK ROM OK should be displayed.	Memory Check: passed	correct
Display and LED Test	A successful test result is shown according to the Method.	LED Function: passed Display Function: passed	correct
Oven heating and temperature stability test	T = 40.X DT = 0.0X STABILITY GOOD is displayed. Displayed temperature on an external digital thermometer: 40 ± 2°C	T = 40.0 DT = 0.02 STABILITY GOOD Displayed temperature on the digital thermometer: 40.0°C	correct

Oven Cooling Test	(Ambient minus [5.0]°C) ± 3°C	Ambient Temperature: 23°C Oven Temperature (digital thermometer): 19.1°C	correct
Leak Sensor Test	The message: LEAK SENSOR TEST SENSOR GOOD is displayed.	Leak Sensor Check: passed	correct
SPD-10AVP:			
Firmware Version Verification	Version is 5.24.	Version: 5.24	correct
Operational Initialization Test	[CHECK GOOD] is displayed after the test.	Initialization: passed	correct
Time Program Test	A successful test result is indicated in the Method.	Time Program: passed	correct
Lamp Energy Test	D2 lamp energy at 220 nm > [800]	D2 lamp energy at 220 nm = 1169	correct
Auto Zero Test	Monitored signal after autozero is within specifications. Absorbance variation: +0.001/-0.00	Auto Zero Function: passed	correct

Wavelength Accuracy (using a mercury lamp as reference standard)	Wavelength peaks at 254 nm (± 2.0 nm)	Wavelength mV Peak 250 303 251 378 252 498 253 536 254 557 255 472 256 401 257 253 258 184 259 73 260 0	correct
RF-10AXL:			
Diagnostic for ROM and RAM	[PASSED] is displayed after the test	Initialization: passed	correct
ROM Version	Version: [3.10] or later	Version: 3.32	correct
Initialization Test	Successful completion of system initialization	Initialization: passed	correct
Wavelength Accuracy (Emission) (using a mercury lamp as reference standard)	253.7 nm ± 2.0 nm 365.0 nm ± 2.0 nm 435.8 nm ± 2.0 nm 546.1 nm ± 2.0 nm	Mercury Line Actual Deviation Wavelength 253.7nm 253.6nm -0.1nm	correct

		365.0nm 364.5nm -0.5nm 435.8nm 434.8nm -1.0nm 546.1nm 545.3nm -0.8nm	
Wavelength Accuracy (Excitation) (using a mercury lamp as reference standard)	253.7 nm ± 2.0 nm 365.0 nm ± 2.0 nm 435.8 nm ± 2.0 nm 546.1 nm ± 2.0 nm	Mercury Line Actual Deviation Wavelength 253.7nm 253.3nm -0.4nm 365.0nm 365.3nm 0.3nm 435.8nm 435.4nm -0.4nm 546.1nm 545.7nm -0.4nm	correct
Sensitivity and S/N Test	Intensity of Raman scattering: 30 - 75 S/N ratio > 300	Raman Intensity Actual Intensity 30-75 74.4 S/N Ratio Actual S/N Ratio > 300 718.3	correct
LC-10A(VP) System:			
System Communication Test	A successful test result is shown according to the Method.	A Pump: passed B Pump: passed Column Oven: passed	correct

		Detector A: passed Detector B: passed Auto Injector Rinse: passed	
Reproducibility Test	Verify that the system meets the acceptance criteria for the %RSD which are shown below: Retention time (Anthracene) < 0.5% Peak area (Anthracene) < 1.0% (2% for RF-detector) Retention time (Pyrene) < 0.5% Peak area (Pyrene) < 1.0% (5% for RF-detector)	SPD-10Avp RF-10AXL Retention time %RSD: Anthracene 0.158 0.152 Pyrene 0.320 0.226 Peak area %RSD: Anthracene 0.491 0.223 Pyrene 0.561 0.948	correct
Gradient System Test: HIGH PRESSURE	Deviation of %Eluent B < 1.0%	%Eluent B: mVolt: %Deviation: 100% 464.9 0 90% 422.9 +0.96 50% 232.3	correct

		-0.04 10% 44.6 -0.41	
Gradient System Test: LOW PRESSURE	Deviation of %Eluent B < 2.0%	%Eluent B: mVolt: %Deviation: 100% 462.2 0 90% 418.3 +0.5 50% 232.5 +0.3 10% 45.1 -0.24	correct

Performance Qualification

The acquired HPLC-device was used for the chromatographical separation and purification of proteins and glycans. Therefore reproducible retention times and peak areas in the chromatograms were of particular interest.

The accomplishment of HPLC-runs with a representative reference substance and the corresponding examination of repeatability of retention times and peak areas was therefore used as verification that the device works properly under "real conditions" and that retention times and peak areas of unknown substances were also repeatable.

To simulate the "real conditions" as best as possible, an HPLC-column for glycosylation analysis was used, namely the GlycoSepC-column. For a better standardization UMP-5' (uridine monophosphate) was used for the PQ-runs. The standard deviation (sdv) of the retention times and the peak areas of the UMP-5'-peak should be used as acceptance criteria for the PQ. For determining the ranges of these standard deviations prequalification studies (not shown) were performed.

Material and methods:

Chromatography column: GlycoSep™ C HPLC Column

Specifications:

Base matrix:	5 µm polymer coated divinyl benzene resin
Derivatization:	DEAE weak anion exchanger
Column dimensions:	4.6 mm * 100 mm
Column volume:	1.7 ml
Typical flow rate:	0.3 – 0.5 ml/min
pH compatibility:	pH 1 to pH 13
Solvent compatibility:	Acetonitrile, methanol, isopropanol

	Avoid strong oxidants and anion detergents.
Typical buffers:	Water/acetonitrile with ammonium acetate or formate buffer
Column tube:	Stainless steel, Valco compatible end fittings
Maximum Pressure:	3000 psi ≈ 206 bar ≈ 20,6 MPA
Chromatography device:	QC-HPLC
Eluent:	0.01 M Tris-HCl (pH 8,0)
Reference substance:	Uridine monophosphate (UMP-5') (FLUKA Biochemika Productno. 94352) Concentration of solution: 2.4 mg/ 1000 µl Injection volume: 1 µl
Method:	Isocratic Flow rate 0.5 ml/min Oven temperature 25°C UV-detection at 254 nm Run time 60 min
Acceptance criteria:	sdv - Retention time [min]: 0.1 sdv - UMP-5'-peak area [Units]: 200000

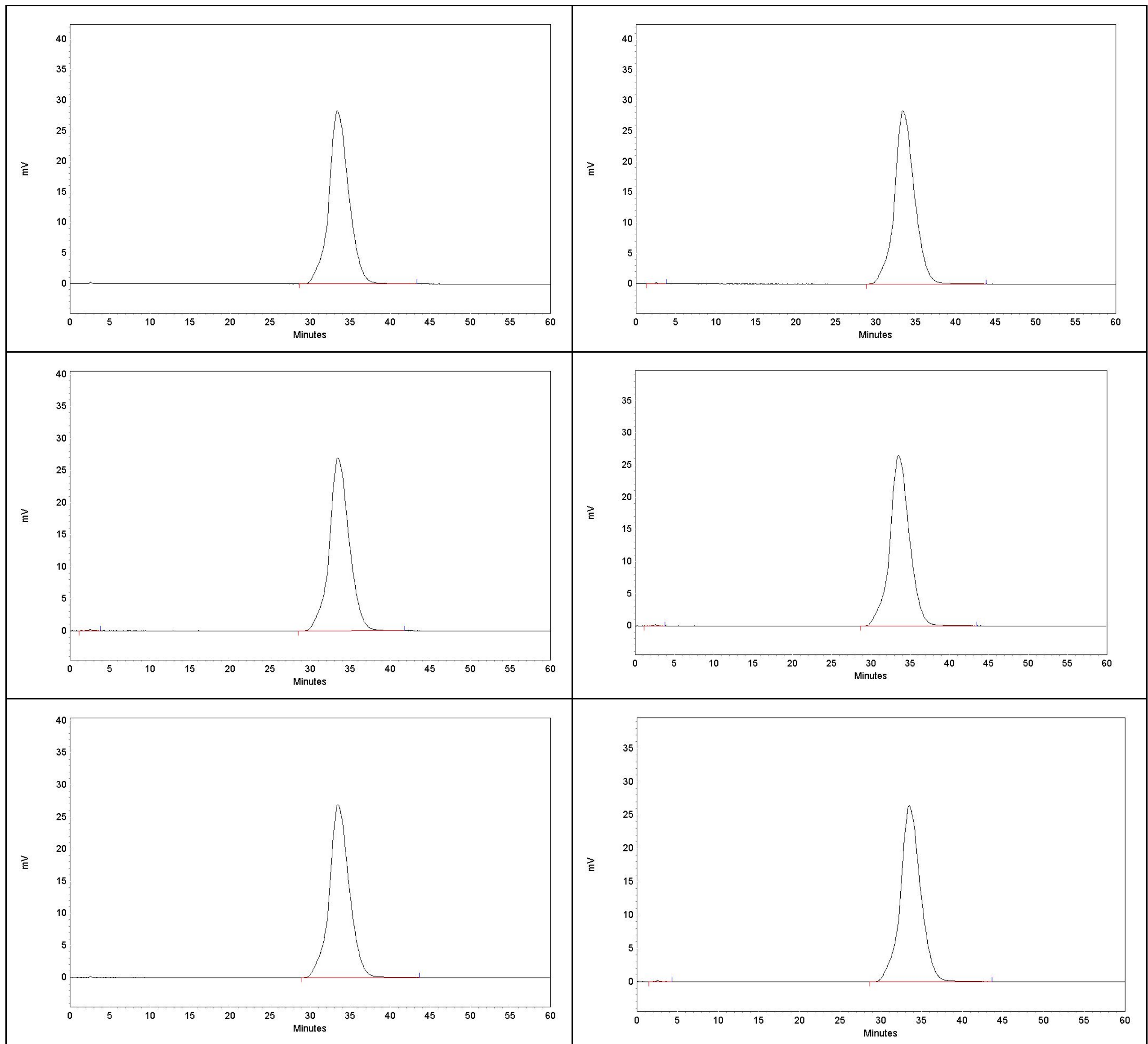
Results:

Six HPLC-runs were performed consecutively. The retention times of the peaks depended very much on the eluents and the age of the column. Both parameters could not be held constant. Therefore only standard deviations of the retention times of the standard peak (UMP-5') were examined.

The HPLC-runs described above were also used for the examination of the repeatability of peak areas. They were mainly influenced by detector characteristics. Integration of the peaks was performed by algorithms (numeric integration) of the Class-VP-Software.

The resulting chromatograms of the PQ-runs are shown in Table 46.

Table 46: Performance qualification runs of the QC-HPLC-device



For the determination of repeatable retention times and peak areas, the runs above were evaluated:

Run	**Retention time [min]**
1	33.411
2	33.413
3	33.480
4	33.535
5	33.485
6	33.495
Average:	33.470
Standard deviation (sdv):	0.049
Acceptance criteria - sdv:	0.1
Result:	Pass
Run	**Area [U]**
1	4905648
2	4916759
3	4652437
4	4607591
5	4667463
6	4569602
Average:	4719917
Standard deviation (sdv):	152157
Acceptance criteria - sdv:	200000
Result:	Pass

Definition of validation parameters

Specificity [166, 167]

Specificity is the ability to assess the analyte unequivocally in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

- Identification: to ensure the identity of an analyte
- Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related test substances, heavy metals, residual solvents content, etc.
- Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

Specificity of methods should be evaluated during the early stages of development and then regularly be reviewed and re-evaluated.

Accuracy [150, 166, 167]

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

This is sometimes termed trueness.

Several methods of determining accuracy are available:

- application of an analytical procedure to an analyte of known purity (e.g. reference material);

- comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure);
- accuracy may be inferred once precision, linearity and specificity have been established.

It is often difficult to determine accuracy of an analytical method by comparison to a second method, especially in the early development phase, where it is rare to have more than one assay and impurity detection method available. Instead accuracy can be inferred from the precision, linearity and specificity studies. In addition, it may be useful to assess the overall mass balance of the analyte during the analytical scheme to verify accuracy of these methods.

Precision [166, 167]

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation, coefficient of variation (relative standard deviation) or confidence interval of a series of measurements.

Repeatability [150, 166, 167]

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Repeatability should be assessed using:

- a minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations/3 replicates each)

or

- a minimum of 6 determinations at 100% of the test concentration.

In early development phase, repeatability of the method should be assessed by performing three sample preparations at 100% of the test concentration.

Intermediate precision [150, 166, 167]

Intermediate precision expresses within-laboratory-variations: different days, different analysts, different equipment, etc.

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

At the early stages of development, it is not necessary to determine the intermediate precision of a method.

Reproducibility [166, 167]

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

Detection limit [150, 166, 167]

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value.

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental one. Approaches other than those listed below may be acceptable.

In early development, it is not crucial to have a defined detection limit since it is sufficient to verify that the reporting limit can be quantified.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways, for example:

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

Quantification limit [166, 167]

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Several approaches for determining the quantification limit are possible, depending on whether the procedure is a non-instrumental or instrumental one. Other approaches than those listed below may be acceptable.

Regardless of the phase of development, the quantification limit for an analytical method should not be greater than its reporting limit.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantification limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantification limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways, for example:

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The quantification limit and the method used for determining the quantification limit should be presented. If QL is determined based on visual evaluation or based on signal-to-noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantification limit.

Linearity [150, 166, 167]

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

A linear relationship should be evaluated across the range of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

For the establishment of linearity, a minimum of 5 concentrations is recommended.

To assess a linear regression line **y = b*x + a** and a correlation coefficient **r**, the following calculations are necessary.

Calculation of linear regression parameters:

$$b = \frac{\sum \left(x_i - \bar{x} \right) \cdot \left(y_i - \bar{y} \right)}{\sum \left(x_i - \bar{x} \right)^2} \qquad a = \bar{y} - b\bar{x}$$

$$r = \frac{\sum \left(x_i - \bar{x} \right)\left(y_i - \bar{y} \right)}{\sqrt{\sum \left(x_i - \bar{x} \right)^2 \cdot \left(y_i - \bar{y} \right)^2}}$$

$x_i = concentrations$

$\bar{x} = average\ of\ concentrations$

$y_i = responses$

$\bar{y} = average\ of\ responses$

For early phase methods, the linear range can be evaluated by ensuring proper quantification at three sample concentrations.

Range [166, 167]

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

The following minimum specified ranges should be considered:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;
- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form, is justified;
- for dissolution testing: +/- 20% over the specified range;
- for the determination of an impurity: from the reporting level of an impurity to 120% of the specification;
- if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

Robustness [150, 166, 167]

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations used are:

- stability of analytical solutions,
- extraction time

In the case of liquid chromatography, examples of typical variations are

- influence of variations of pH in a mobile phase,
- influence of variations in mobile phase composition,
- different columns (different lots and/or suppliers),
- temperature,
- flow rate.

During early development robustness testing can be limited to demonstrating that solutions are adequately stable for their duration of use in the lab.