THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

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Reference standards for microbiological assay of antibiotics

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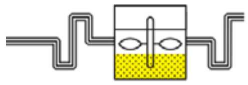
Antibiotics in Ph. Eur. monographs

Production process of antibiotics

- Synthetic
- Semi-synthetic
- Fermentation



Single compound
Mixture of compounds

Specific quality requirements

Related substances

- limit for unspecified impurities
- total impurities
- disregard limit

Assay

- LC: content in %m/m determined against a CRS with assigned value
- microbiological: different type of RS and arbitrary units valid wordwide

Ph. Eur. 2.7.2.: Microbiological assay of antibiotics

- The **potency** of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and the corresponding **reference substance (CRS)**
- **Recommended** micro-organisms: other may be used provided they are shown to be suitable
- Preparation of inocula, buffer solutions, culture media composition are described

Table 2.7.2.-1. – *Diffusion assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Amphotericin B	*Amphotericin B for microbiological assay CRS*	*Dimethyl sulfoxide R*	pH 10.5 (0.2 M)	*Saccharomyces cerevisiae* ATCC 9763 IP 1432-83	F - pH 6.1	35-37 °C
Bacitracin zinc	*Bacitracin zinc CRS*	*0.01 M hydrochloric acid*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 7743 CIP 53.160 ATCC 10240	A - pH 7.0	35-39 °C
Bleomycin sulfate	*Bleomycin sulfate CRS*	*Water R*	pH 6.8 (0.1 M)	*Mycobacterium smegmatis* ATCC 607	G - pH 7.0	35-37 °C
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
				Escherichia coli NCIMB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Colistin sulfate	*Colistin sulfate for microbiological assay CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
				Escherichia coli NCIMB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C

Ph. Eur. 2.7.2.: Methods

Method A: Diffusion assay

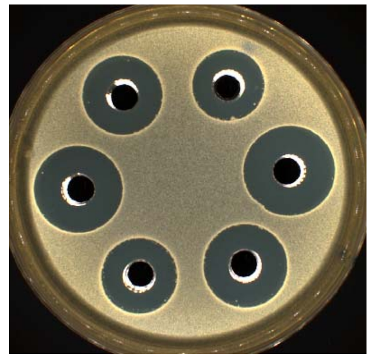
- Inoculated medium is poured into Petri dishes
- Reference and tests solutions are applied to the wells made in the agar
- Dishes are incubated for about 18h
- The antibiotic creates an inhibition zone around the well
- The diameter of the inhibition zone is measured

HUMPHREY, J. H. & LIGHTBOWN, J. W. (1952). *J. gen. Microbiol.* 7, 129-143

A General Theory for Plate Assay of Antibiotics with some Practical Applications

BY J. H. HUMPHREY AND J. W. LIGHTBOWN

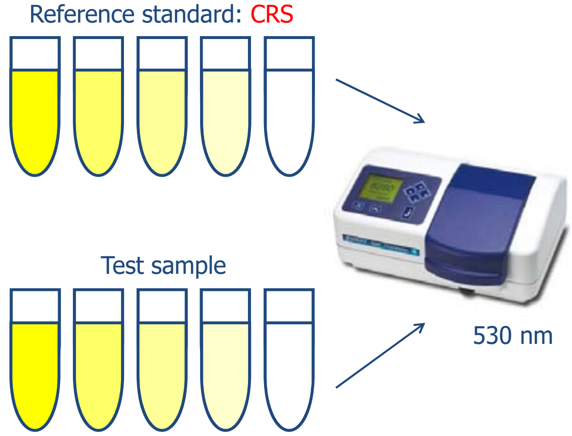
National Institute for Medical Research, Mill Hill, London, N.W. 7



Ph. Eur. 2.7.2.: Methods

Method B: Turbidimetric assay

- Reference and test solutions are distributed into test tubes
- Inoculated medium is added
- Tubes are incubated in a water-bath for about 4h
- Turbidimetry of the medium in each test tube is read by measurement with a spectrophotometer
- Turbidimetry is inversely proportional to the concentration of the antibiotic



Microbiological assay of antibiotics: other Pharmacopoeias

- **Ph. Eur.** general chapter
  2.7.2. Microbiological assay of antibiotics
- **USP** general chapter
  <81> Antibiotics – Microbial assays
- **JP** general chapter
  4.02 Microbial assay for antibiotics
- **International Pharmacopoeia** general chapter
  3.1 Microbiological assay of antibiotics

→

- Same methods described: diffusion, turbidimetry
- Procedure highly similar, design may differ
- Same intended purpose of reference standard
- Slight differences in the antibiotics listed

Antibiotic assay units

- RS for antibiotic assay may be expressed in:

International system of units (SI)

mg

- applicable to chemically homogeneous (pure) substances
- CRS established using mass balance approach based on monograph methods
- traceable to a higher order standard

-> content

LC improved ←

Arbitrary units

International Units

- essential for substances of complex and heterogeneous chemical structure
- unitage assigned by WHO
- traceable to a higher order standard

-> potency
determined by measuring antibiotic inhibitory effect on a microorganism

Other arbitrary units

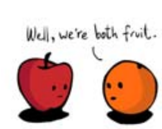
"unit" of antibiotic

- more than one active compound in the antibiotic

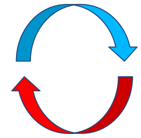
"µg" of activity

- thought to consist of a single chemical entity

Discrepancy in arbitrary units:
- legacy of the past
- questioned by users
- risk of misuse of RS and units



Lost in conversion



- **Principle:**
  - o quantity of substance is measured in mass
  - o potency is estimated in units defined by a reference standard

-> potency of complex antibiotics (mixtures) cannot be measured in terms of mass

- **Definition of IU:**
  - o activity contained in a given amount (mg or vial) of a particular batch of a reference standard expressed in an assay system -> **≠ mass unit**
  - o IU depends on the activity of the substance and therefore varies from substance to substance

Example: 1st ISA for Gentamicin
The IU was defined in 1968 as the activity contained in 0.00156 mg of the preparation

1 IU ≠ 0.00156 mg and 1 IU ≠ 1.56 µg
but in this case 1 IU = 1"µg" of activity

-> Do not use conversion factor
Use the RS established for the intended purpose in the corresponding Pharmacopoeia

RS for microbiological assay of antibiotics at EDQM

Primary standard: International Standard for Antibiotic (ISA)
- o Use: establishment of national/regional secondary standards
- o Established, kept, distributed by EDQM, approved by WHO

Secondary standard: Ph. Eur. CRS for microbiological assay of antibiotic
- o Use: routine quality control
- o Established by EDQM against the ISA, approved by the Ph. Eur. Commission

➡ Advantages of Ph. Eur. CRS:
Traceability is ensured
Same unitage: International Unit (IU)

RS for microbiological assay of antibiotics: our portfolio

Primary standard: 23 International Standard for Antibiotic (ISA)	
Amphotericin B	Netilmicin
Bacitracin	Nystatin
Bleomycin complex A2/B2	Polymyxin B
Colistin	Rifamycin SV
Colistin Methane Sulfonate	Sisomicin
Dihydrostreptomycin	Spiramycin
Erythromycin	Streptomycin
Gentamicin	Teicoplanin
Gramicidin	Tobramycin
Kanamycin	Tylosin
Neomycin	Vancomycin
Neomycin B	

Secondary standard: 22 Ph. Eur. CRS for microbiological assay of antibiotic	
Amphotericin B for microbiological assay	Kanamycin Monosulfate
Bacitracin Zinc	Neomycin Sulfate for microbiological assay
Bleomycin Sulfate	Netilmicin Sulfate
Colistin Sulphate for microbiological assay	Nystatin
Colistimethane sodium	Polymyxin B Sulfate for microbiological assay
Erythromycin for microbiological assay	Rifamycin Sodium
Framycetin Sulfate	Spiramycin
Gentamicin Sulfate	Streptomycin Sulfate
Gramicidin	Teicoplanin
Josamycin	Tylosin
Josamycin propionate	Vancomycin Sulfate

➡ Established by collaborative studies

Transitioning from microbiological assay to LC in Ph. Eur.

Introduction of LC can be envisaged when:
- **purity** of antibiotic is high e.g. > 90 %
- structure of the substance is known
- selective and accurate chromatographic methods are available

Examples in the Ph. Eur.:

Antibiotic	CRS for LC assay	CRS for microbiological assay	ISA
Tobramycin	yes	no	yes
Erythromycin	yes	yes	yes
Dihydrostreptomycin sulfate	yes	no	yes
Netilmicin sulfate	yes	yes	yes

Re-introducing microbiological assay

Challenges of LC assay for mixtures:

- preparation and the establishment of the required reference substance can be technically difficult
- biological activity of the different physicochemical entities might not be identical

Example: mixture of polypeptide sulfates

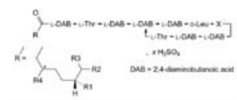
POLYMYXIN B SULFATE

Polymyxini B sulfas

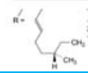
Polymyxin	R	R'	X	Molecular formula	M_r
B1	CH_3	CH_3	L-Leu	[illegible]	1203
B2	H	CH_3	L-Leu	[illegible]	1189
B3	CH_3	H	L-Leu	[illegible]	1189
B1-I	CH_3	CH_3	L-Ile	[illegible]	1203

COLISTIN SULFATE

Colistini sulfas



polymyxin	X	R1	R2	R3	R4	Mol. Formula	M_r
E1	L-Leu	CH_3	CH_3	H	H	[illegible]	1169
E2	L-Leu	CH_3	H	H	H	[illegible]	1155
E3	L-Leu	H	CH_3	H	H	[illegible]	1155
E4	L-Leu	H	H	H	H	[illegible]	1141
E6	L-Leu	CH_3	CH_3	H	OH	[illegible]	1185
E1-7MOA	L-Leu	H	CH_3	CH_3	H	[illegible]	1169
E1-I	L-Ile	CH_3	CH_3	H	H	[illegible]	1169
E1-Nva	L-Nva	CH_3	CH_3	H	H	[illegible]	1155
E2-I	L-Ile	CH_3	H	H	H	[illegible]	1155
E2-Val	L-Val	CH_3	H	H	H	[illegible]	1141



polymyxin	X	Mol. Formula	M_r
2,3-dehydro E1	L-Leu	[illegible]	1167

-> In view of difficulties with the expression of the content of the substance after replacement of the microbiological titration by an LC assay, **the microbiological titration has been re-introduced**

Perspective

- Widespread and increasing resistance to antibiotics worldwide
- New antibiotics in development: it is not known if they are effective against the most dangerous forms of antibiotic-resistant bacteria
- Improvement of existing antibiotics and acceleration of the entry of new antibiotic drugs needed
- Recent recommendation that current WHO listing of international standards for antibiotics be reviewed

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