UNIVERSITÄT WÜRZBURG

Institut für Pharmazie und Lebensmittelchemie

Das Arzneibuch und orthogonale Methoden

- NMR-Spektroskopie -

Ulrike Holzgrabe

Chair of the working group
„Arzneimittelsicherheit/Arzneimittelfälschung“
of the German Pharmaceutical Society (DPhG)
University of Würzburg

UNIVERSITÄT WÜRZBURG

Counterfeit drugs

4 Catagories of counterfeits:

- Mislabelled counterfeits

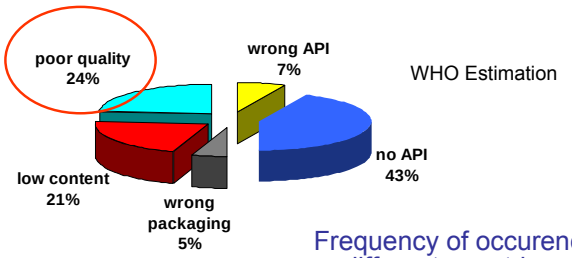
Ciprobay against anthrax shortly after 11th September 2001

Tamiflu® counterfeits in expectation of a influenza pandemia? May 2007

- Counterfeits containing less API
- Counterfeits containing the wrong API
- Counterfeits containing no API

UNIVERSITÄT WÜRZBURG

WHO Counterfeits (2006)



WHO Estimation

Frequency of occurence in different countries

USA	5 - 7 %
GUS	> 20 %
Africa	50 – 60 %
Mexico Latin America South East Asia	30 %

German Pharma Health Fund eV.

UNIVERSITÄT WÜRZBURG

The 5th category

drugs of substandard quality

Active pharmaceutical ingredients (API) consisting of

- New related substances or high amounts of old and/or new related substances
- High amounts of residual solvents
- High amounts of heavy metals

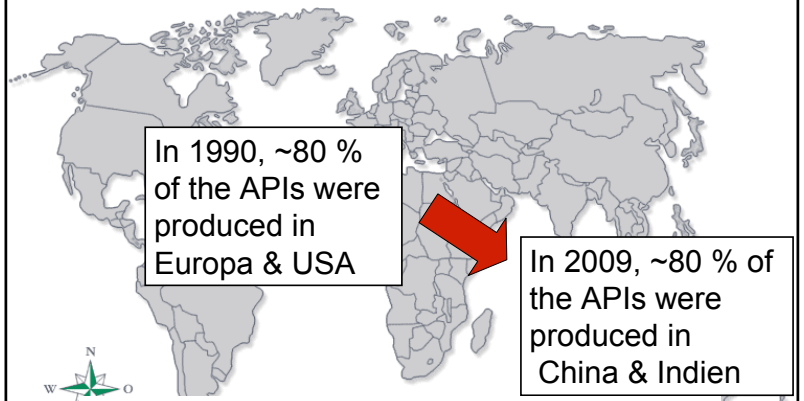
Due to changes in synthesis, extraction or purification (~ 25 % of counterfeit drugs)

Deaths caused by drugs of inaedaquate purity

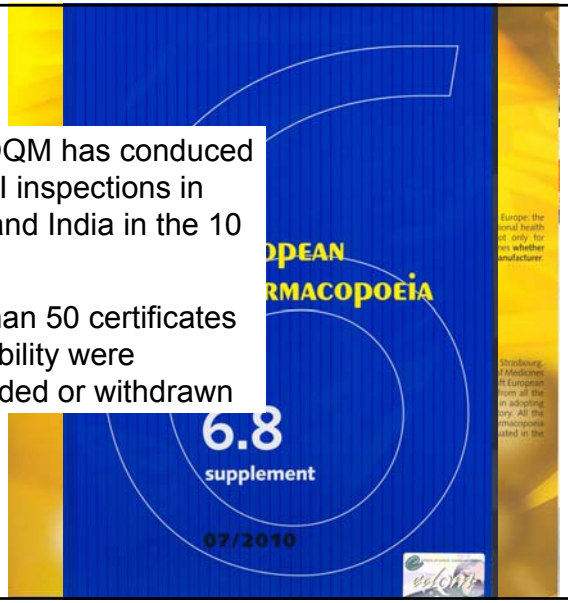
- 1990 Paracetamol-syrup contaminated with diethylene glycol originated from glycerol (some 80 deaths)
- 1990 Tryptophan-Affair: the change in the production process led to additional impurities causing the Eosinophilia-Myalgia-Syndrome (EMS) some 30 deaths
- 2000 Gentamicin in high concentrations (off-label-use) caused the death of some 60 people in the USA
- 2007/8 Heparin with anaphylactoid, oversulfated chondroitin sulfate, approx. 100 deaths in USA
- 2006-9 Diethylene glycol in glycerin from China: dozens of deaths in Panama, in tooth paste in UK, in syrup for teething children in Nigeria

5

Shift of production plants



Problem: low number of inspections in China!



The EDQM has conduced 160 API inspections in China and India in the 10 years:

More than 50 certificates of suitability were suspended or withdrawn

7

The situation is not as good as claimed by the EDQM.

- Not all monographs are as modern as they should be.
- There are ways to circumvent the purity control = non detected impurities

Orthogonal methods are necessary!

qNMR, Raman, IR spectroscopy, other separation techniques than HPLC, e. g. capillary electrophoresis

8

Outline

- Fundamentals
- The gentamicin case
- Diethylene glycol in glycerin
- qNMR of Heparin: OSCS, dermatan and more
- Conclusion

Outline

- Fundamentals
- The gentamicin case
- Diethylene glycol in glycerin
- qNMR of Heparin: OSCS, dermatan and more
- Conclusion

NMR is a Primary Method

Comité consultatif pour la quantité de matière (CCQM)

Definition April 2000:

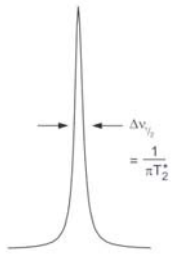
A **primary method** of measurement is a method having the highest metrological properties, whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of SI units

- A **primary direct method** measures the value of an unknown without reference to a standard of the same quality
- A **primary ratio method** measures the value of a ratio of an unknown to a standard of the same quantity; its operation must be completely described by a measurement equation.

B. King, Accredit. Qual. Assur. 5 (2000) 266-271 & 429-436

NMR spectroscopy in quantitative analysis

The intensity of a NMR-signal is given by the area under the specific signal. The intensity *I* of a signal is directly proportional to the number of nuclei *N* evoking that signal. The relation between the signal intensity *I* and the number of observed nuclei is given by: $I = c_S \cdot N$



Integration from -∞ until +∞

Relative method:
molar ratio

$$\frac{n_X}{n_Y} = \frac{I_X}{I_Y} \cdot \frac{N_Y}{N_X}$$

Absolute method:

Main component P_x

$$P_X = \frac{I_X}{I_{Std}} \cdot \frac{N_{Std}}{N_X} \cdot \frac{M_X}{M_{Std}} \cdot \frac{m_{Std}}{m} \cdot P_{Std}$$

Factors to be considered

Precision of the integrals determines the accuracy of quantification

- depends on the noise level of the spectrum
- depends on the line shape
- quality of shimming
- choice of the window function
- phase-, baseline- and drift corrections (user!)
- relaxation: determination of T_1 of the signals considered**!**
- integral interval (64 times the full width at half signal height)

⇒ Optimization and validation of the method is necessary

Diehl, Malz, Holzgrabe, Spectroscopy Europe 17 (2007) 5: 15 – 19;
Holzgrabe: Progr. NMR spectroscpy 57 (2010) 229-240

Problems to Overcome

$S/N = \sqrt{n} \cdot (S/N)$

- Sensitivity
  - high field strength,
  - invers and (micro) cryo probes,
  - the higher number of scans (square roots on N!) the higher the S/N ratios: > 250:1 for ^{1}H, > 300:1 for ^{19}F and > 600:1 for ^{31}P are necessary for sdv < 1%,
  - gradient shimming (quality)
- Signal overlap (signal purity)
  - choice of the solvent (e.g. ASIS),
  - different sample concentrations,
  - pH value of the solution, if analytes are basic or acid,
  - addition of auxiliary reagents like cyclodextrins or lanthanide shift reagents,
  - temperature, which also affects the signal separation
- Rotational side bands & ^{13}C satellites

Applications

NMR spectroscopy can be used

- to identify a drug or an excipient
- to evaluate the level of impurities (and to elucidate the structure)
- to observe the course of decomposition
- to evaluate residual solvents
- to determine the isomeric composition:

  the ratio of diastereomers
  the ee by means of chiral additive
- to assess a single drug or drug composition
- to characterize a polymer
- To identify the counterion (organic)
- to unravel counterfeit drugs (e. g. ^{1}H, ^{13}C, ^{19}F NMR or DOSY, NOESY, etc.)

NMR in International Pharmacopoeiae

Identity:	DAB 9:	Gentamicin (^{1}H)
	PhEur 6:	Buserelin (^{1}H), Goserelin (^{13}C), Tobramycin (^{1}H)
		Heparins low-molecular-mass, unfractionated Heparin (^{1}H),
		Vaccines: HaemophilusType b conjugate vaccine,
		Meningococcal Group C conjugate vaccine,
		Pneumococcal polysaccharide conjugate vaccine
		Salmon oil farmed (^{13}C)
	BP 1998:	Hydrocortisone Sodium Phosphate (^{1}H)
	USP 26:	Amylnitrite isomers (^{1}H)
Tests:	DAB 9:	Gentamicin
	PhEur 6:	Poloxamer: ratio of oxypropylene/oxyethylene,
		Hydroxypropylbetadex: molar substitution,
		Lauromacrogol 400: average chain length of fatty alcohol and ethylene oxide (in preparation)
	USP 30:	Orphenadrine citrate: *meta*/*para* isomer
Assay:	USP 30:	Amylnitrite isomers

Outline

- Fundamentals
- The gentamicin case
- Diethylene glycol in glycerin
- qNMR of Heparin: OSCS, dermatan and more
- Conclusion

Gentamicin case

Acute massive gentamicin intoxication **in a patient with end-stage renal disease**
Lu CMC, James SH, Lien YHH
AMERICAN JOURNAL OF KIDNEY DISEASES
28 (5): 767-771 NOV 1996

Document type: Article **Language:** English **Cited References:** 19 **Times Cited:** 0

Abstract:
A 65-year-old man with end-stage renal disease on continuos ambulatory peritoneal dialysis accidentally received an acute massive overdose of **gentamicin** as a treatment of peritonitis. The patient developed acute vestibular dysfunction and hearing loss following the overdose. His serum **gentamicin** had
the patient receiv

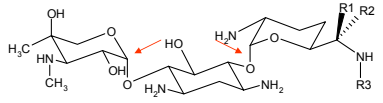
MMWR Weekly October 23, 1998 / 47(41);877-880

Endotoxin-Like Reactions Associated with Intravenous Gentamicin -- California, 1998

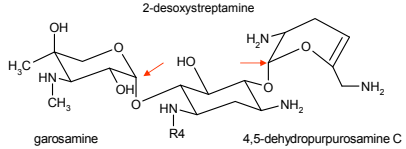
During April 30-July 26, 1998, 20 patients at a major medical center (hospital A) in Los Angeles County, California, developed severe shaking chills often accompanied by fever, tachycardia, and/or a decrease of greater than or equal to 20 mm Hg in systolic blood pressure within 3 hours after receiving intravenous (IV) gentamicin. Receipt of IV gentamicin was the only medication or procedure temporally associated with reactions among all of the patients. No deaths or serious sequelae were associated with the reactions. Similar incidents were reported by hospital personnel from six other states to CDC or the Food and Drug Administration (FDA) during April-August 1998. All reported reactions were associated with once-daily dosing regimens of gentamicin (lot numbers 170704,

Gentamicin case

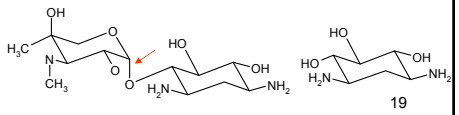
PhEur 6.0: Gentamicin sulfate is a mixture of the sulfates of antimicrobial substances produced by ***Micromonospora purpurea,*** the main compounds being gentamicin **C1**, **C1a**, **C2**, **C2a**, and **C2b**.



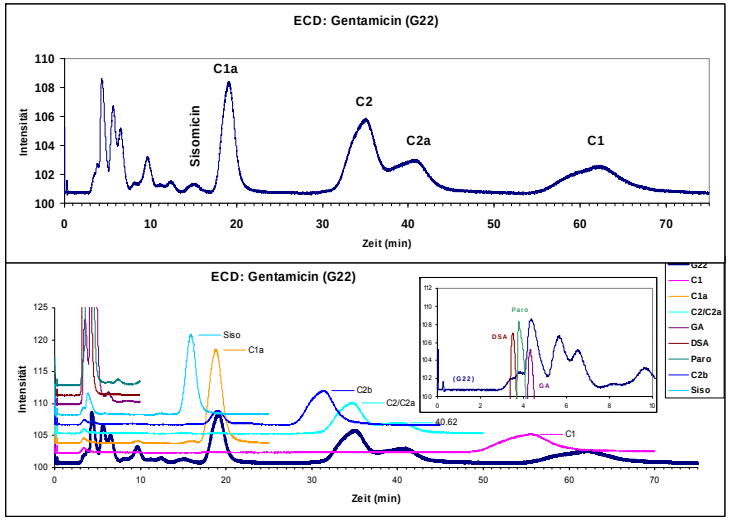
	R1	R2	R3
Gentamicin C1	$-CH_3$	-H	$-CH_3$
Gentamicin C1a	-H	-H	-H
Gentamicin C2	$-CH_3$	-H	-H
Gentamicin C2a	-H	$-CH_3$	-H
Gentamicin C2b	-H	-H	$-CH_3$



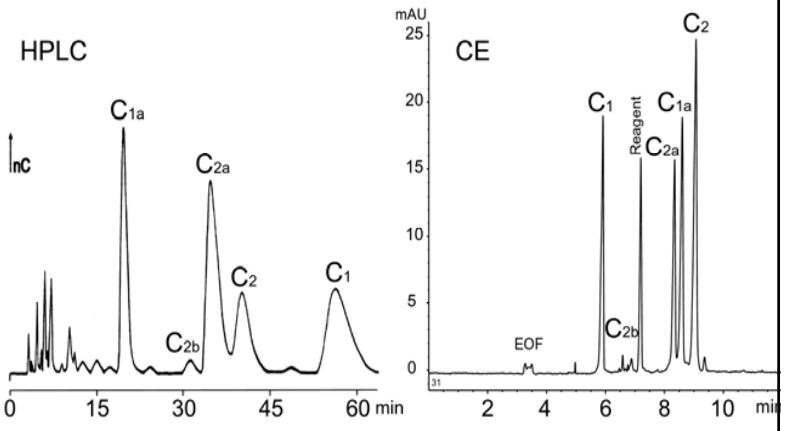
	R4	
Sisomicin	-H	
Netilmicin	$-CH_2-CH_3$	(Pos. 30/31)



HPLC, pulsed amperometric detection



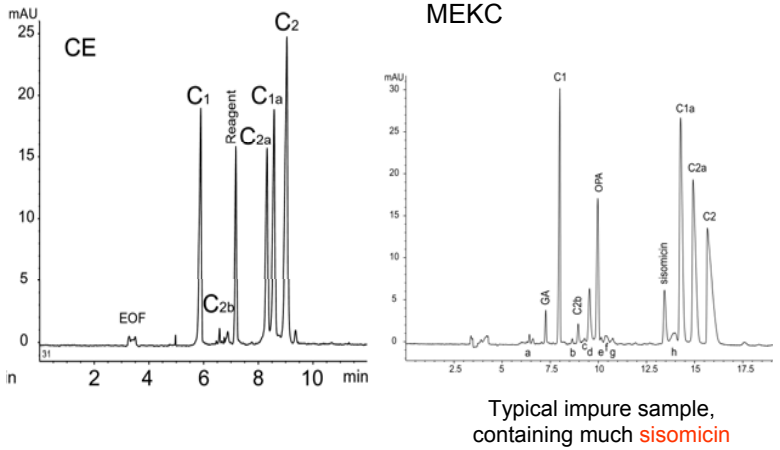
Comparison PhEur HPLC & the CE method



Adams et al. JPBA 18 (1998) 689

Kaale et al. J. Chromatogr. A 895 (2000) 67

Aiming for more selectivity: MEKC



Typical impure sample, containing much sisomicin

Wienen, Deubner, Holzgrabe, Pharmeuropa 15 (2003) 273

Wienen, Holzgrabe, Electrophoresis, 24 (2003) 2948
Validation: Kühn, Weber, Holzgrabe, JPBA 48 (2008) 612

DAB 9 - NMR an orthogonal method

Identity

Das Kernresonanzspektrum (siehe „Prüfung auf Reinheit") einer 20prozentigen Lösung (*m*/V) der Substanz in [D_2]Wasser *R* steht im Einklang mit dem einer Lösung von Gentamicinsulfat *CRS* gleicher Konzentration. (Aufgrund eines verschiedenen Verhältnisses der Einzelkomponenten können Unterschiede bei den Integrationen der Signale bei etwa δ 1,25, 1,35, 2,75 und 2,95 auftreten.)

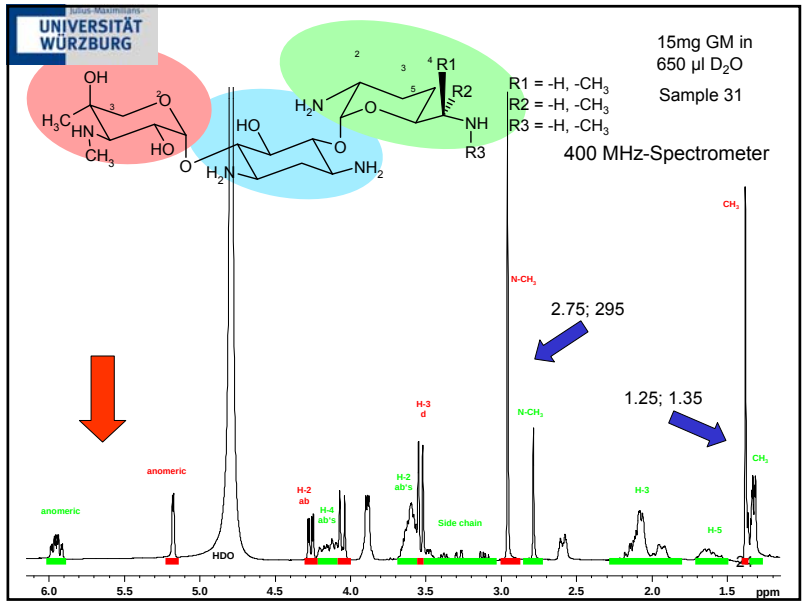
60 MHz NMR spectrometer!

Tests

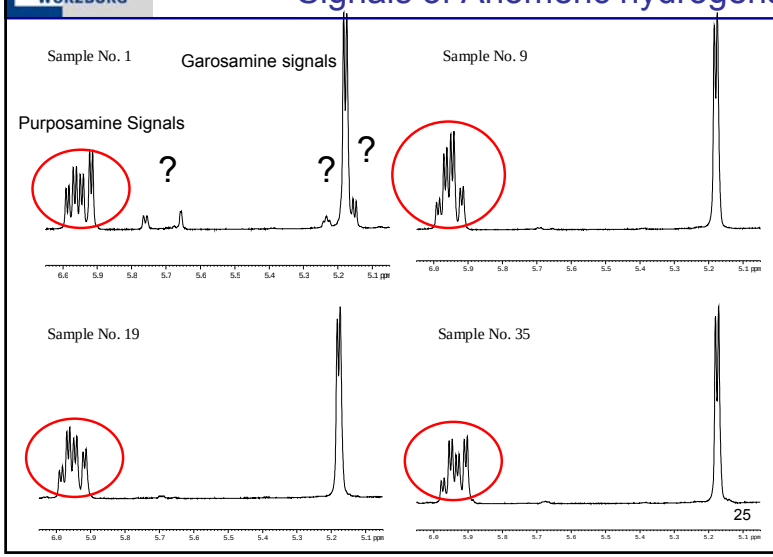
unter den oben angegebenen Bedingungen aufgezeichnet. Falls erforderlich, wird eine erneute Einstellung der Phase und der Empfindlichkeit vorgenommen. Das Verhältnis der Höhe des Signals bei etwa δ 2,75 zu der des Signals bei etwa δ 2,95 muß zwischen 0,260 und 0,440 liegen. Das Verhältnis der Höhe des Signals bei δ etwa 1,25 zu der des Signals bei δ etwa 1,35 muß zwischen 0,200 und 0,260 liegen. Die Messung der Signalhöhen erfolgt von einer Linie aus, die zwischen der mittleren Basislinie zwischen δ 0 und δ 0,5 und der zwischen δ 6,1 und δ 6,6 gezogen wird. Die Verhältnisse sind das arithmetische Mittel der von jedem Spektrum erhaltenen Werte.

D.H. Calam et al. J. Pharm. Pharmacol. 30 (1978) 220

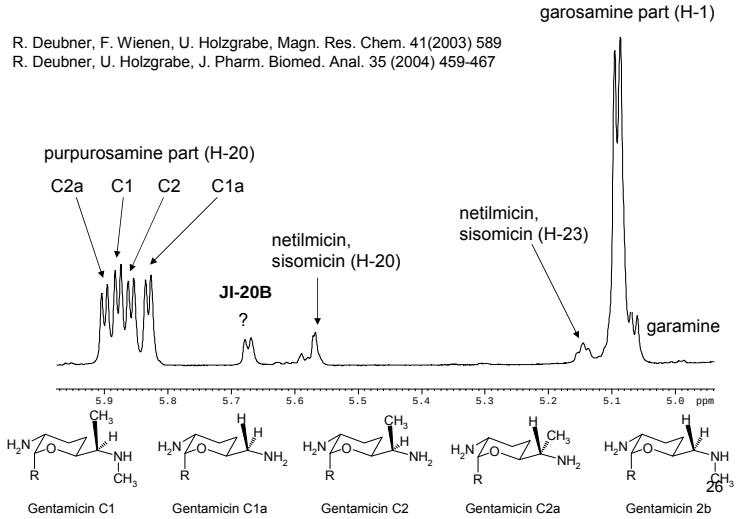
23



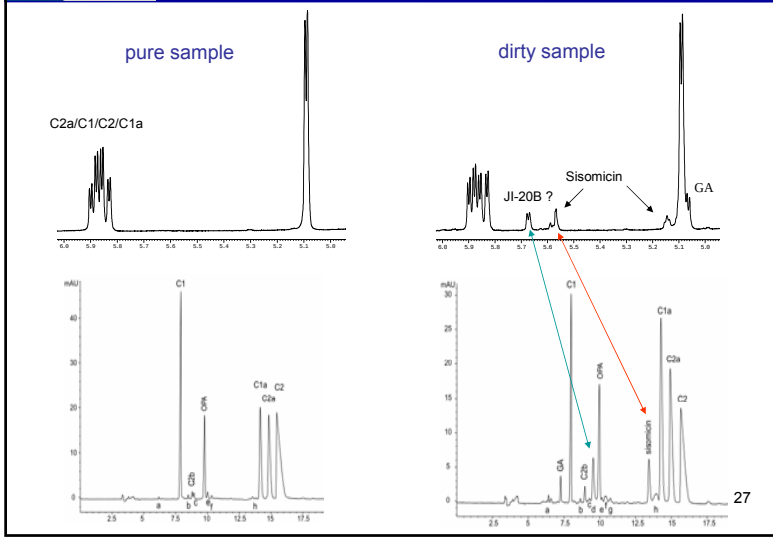
Signals of Anomeric hydrogens



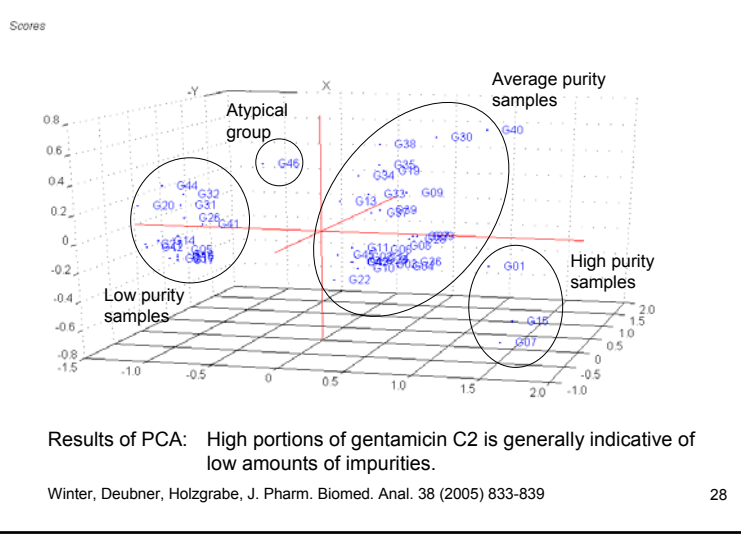
Assignments of the signals



Comparison NMR-MEKC



3D score plot of a PCA model



Results of PCA: High portions of gentamicin C2 is generally indicative of low amounts of impurities.

Winter, Deubner, Holzgrabe, J. Pharm. Biomed. Anal. 38 (2005) 833-839

28

Conclusions - Gentamicin

NMR spectroscopy can be used in impurity profiling!

Gentamicin profiling:

- 8 different composition pattern could be identified
- Some patterns occurred in samples of different origin
- Samples from same sources exhibited different patterns indicating different producers (than known to the MAA holder and known to the authorities)
- "Dirty" samples were found to be the samples which were responsible for deaths and side effects

References:

F. Wienen, R. Deubner, U. Holzgrabe, Pharmeuropa 15 (2003) 273
R. Deubner, F. Wienen, Magn. Res. Chem. 41 (2003) 589
F. Wienen, U. Holzgrabe, Electrophoresis, 24 (2003) 2948
R. Deubner, U. Holzgrabe, J. Pharm. Biomed. Anal. 35 (2004) 459
U. Holzgrabe, R. Deubner, F. Wienen, American Pharmaceutical Outsourcing 5 (2004) 24-29
etc.

Outline

- Fundamentals
- The gentamicin case
- Diethylene glycol in glycerin
- qNMR of Heparin: OSCS, dermatan and more
- Conclusion

Diethylene glycol in glycerin

- *1985 Diethylene glycol in Austrian Wine*
- 1990 Paracetamol-syrup contaminated with diethylene glycol originated from glycerol (some 80 deaths)
- 2006 Diethylene glycol in glycerin from China: dozens of deaths in Panama
- 2007 Diethylene glycol in glycerin found in tooth paste in UK
- 2009 Diethylene glycol in glycerin in syrup for teething children in Nigeria

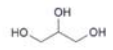
Diethylene glycol in glycerin

01/2008:0496

Ph. Eur. 6.3

GLYCEROL

Glycerolum

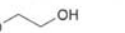


$C_3H_8O_3$

[56-81-5]

IMPURITIES

A. 2,2'-oxydiethanol (diethylene glycol),



B. ethane-1,2-diol (ethylene glycol),

C. propylene glycol.

Every impurity	0.1 %
Total of all	0.5 %

Impurity A and related substances. Gas chromatography (2.2.28).

Test solution. Dilute 10.0 ml of solution S to 100.0 ml with *water R*.

Reference solution (a). Dilute 10.0 g of *glycerol R1* to 20.0 ml with *water R*. Dilute 10.0 ml of the solution to 100.0 ml with *water R*.

Reference solution (b). Dissolve 1.000 g of *diethylene glycol R* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with reference solution (a). Dilute 1.0 ml of this solution to 20.0 ml with reference solution (a).

Reference solution (d). Mix 1.0 ml of the test solution and 5.0 ml of reference solution (b) and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

Reference solution (e). Dilute 5.0 ml of reference solution (b) to 100.0 ml with *water R*.

Column:

- *size:* l = 30 m, Ø = 0.53 mm,
- *stationary phase:* 6 per cent polycyanopropylphenyl siloxane and 94 per cent of polydimethylsiloxane.

Carrier gas: *helium for chromatography R*.

Split ratio: 1:10.

Linear velocity: 38 cm/s.

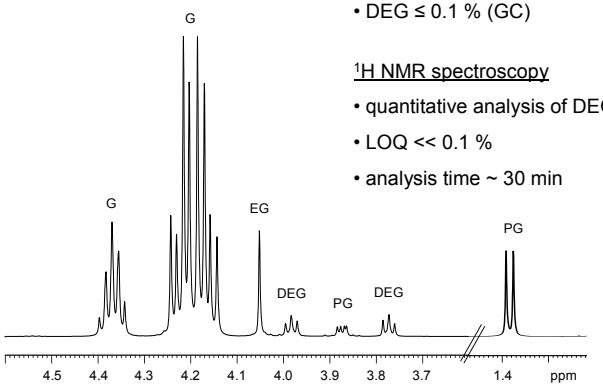
Temperature:

	Time (min)	Temperature (°C)
Column	0 0 - 16 16 - 20	100 100 → 220 220
Injection port		220
Detector		250

Detection: flame ionisation.

Diethylene glycol in glycerin

Purity control of glycerin by means of ^{1}H NMR spectroscopy



^{1}H NMR spectrum of glycerin contaminated with 4 % DEG, EG and PG (400 MHz, pyridine-d_5, 128 scans).

Limit of related substances in PhEur 6.0

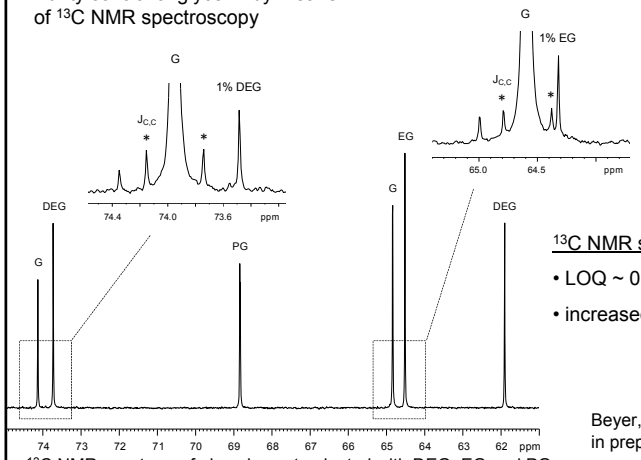
- DEG ≤ 0.1 % (GC)

^{1}H NMR spectroscopy

- quantitative analysis of DEG, EG and PG
- LOQ << 0.1 %
- analysis time ~ 30 min

Diethylene glycol in glycerin

Purity control of glycerin by means of ^{13}C NMR spectroscopy



^{13}C NMR spectroscopy

- LOQ ~ 0.1 %
- increased analysis time

Beyer, Holzgrabe, in preparation

^{13}C NMR spectrum of glycerin contaminated with DEG, EG and PG (400 MHz, pyridine-d_5, 512 scans).

Outline

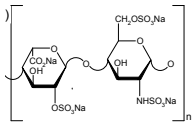
- Fundamentals
- The gentamicin case
- Diethylene glycol in glycerin
- qNMR of Heparin: OSCS, dermatan and more
- Conclusion

Heparin Case

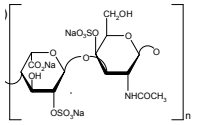
The History

new anion-exchange HPLC and a new biological assay were introduced in the test section, etc.

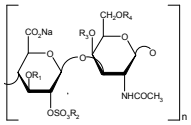
Heparin Case



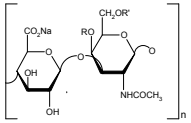
Heparin
Glucosamine + Iduronic acid,
N-acetylated Glucosamine + Glucuronic acid



Dermatan sulfate
Galactosamine + Iduronic acid

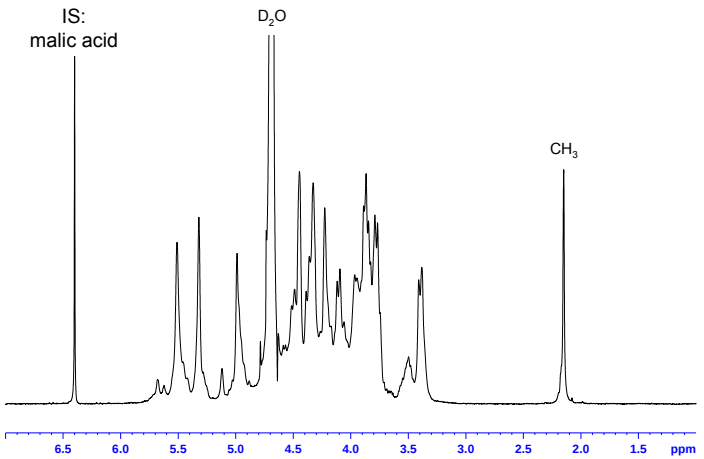


„oversulfated" Chondroitin sulfate
Galactosamine + Glucuronic acid
R_1-R_4 = sulfated
OSCS



Chondroitin sulfate A/C
Galactosamine + Glucuronic acid
R = R´ = sulfated

Heparin - ^{1}H NMR



pure Heparin, D_2O, 400 MHz, 315K

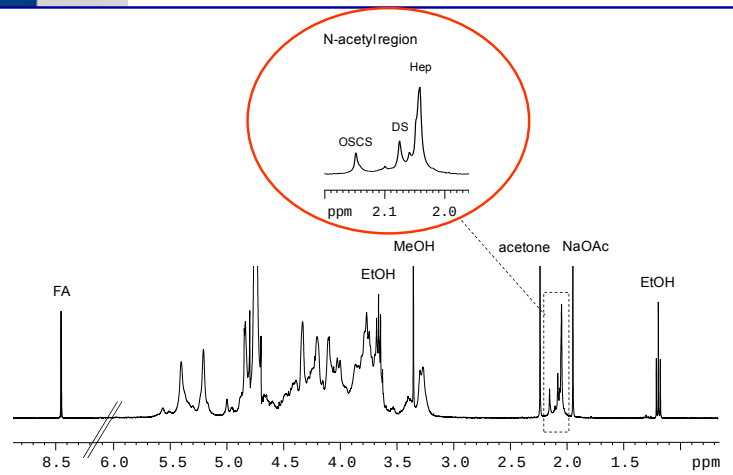
Heparin Cases

What can be done by means of NMR spectroscopy?

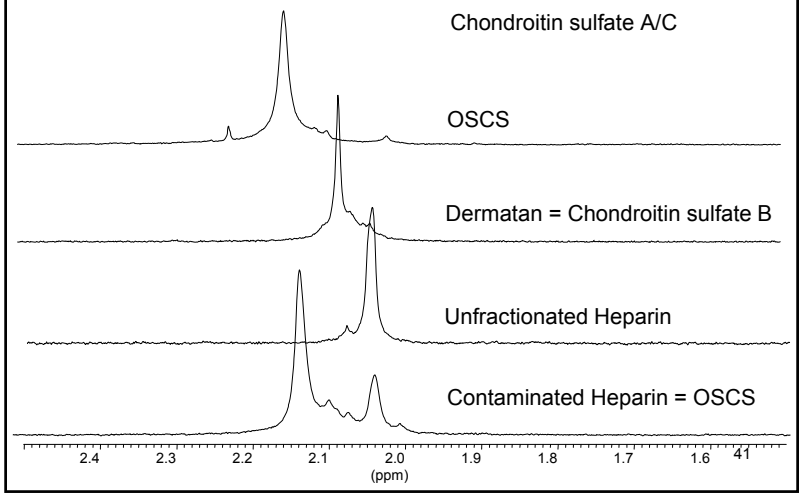
Test on Identity
Heparin
Chondroitin Sulfate A and C
Dermatan Sulfate
OSCS (A/C)
OSDS
Hyaluronic acid
Determination of Animal Source at 353 K

Amount	Quantification by internal standard	
by-products	Dermatan Sulfate	LOD approx. 0.5%
Contamination	OSCS	LOD approx. 0.1%
Solvent residue	Methanol, Ethanol, DMF	LOD approx. 50 ppm dir.
Drug Products	By water suppression	
LMW Heparine	Enoxiparin, Fraxiparin, etc	

Heparin ^{1}H NMR spectrum

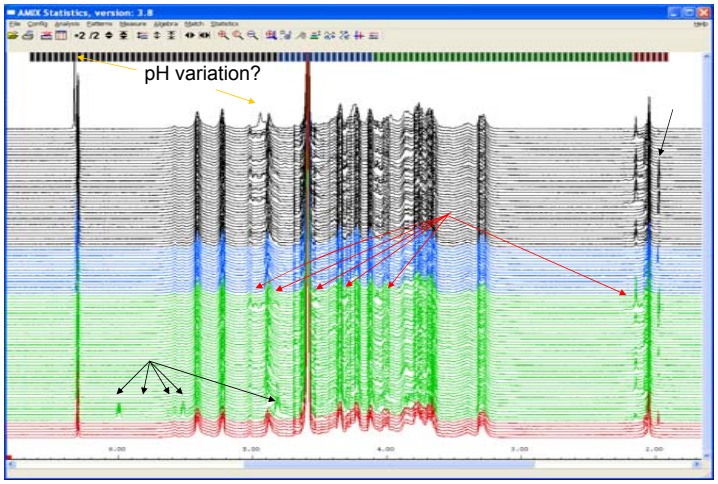


Heparin - ^{1}H NMR



Heparin - ^{1}H NMR

pH variations and content of Ca^{2+} and Mn^{2+}

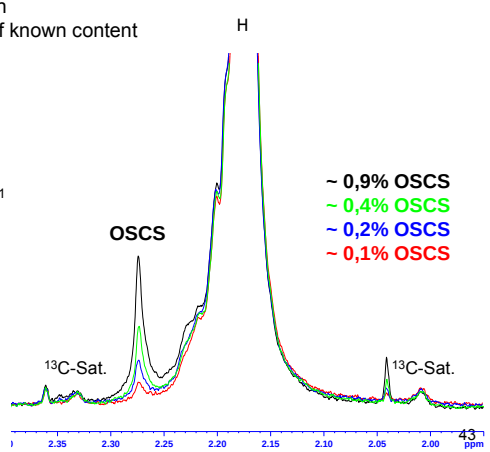


Heparin – LOD NMR

1) pure Heparin spiked with OSCS
2) pure Heparin spiked with
contaminated Heparin of known content

Experimental parameters
- 400 MHz
- 90°-Puls (*versus* 30°)
- NS = 128
- T_1 (CH_3) = 1,44s → 5 x T_1
- T = 315K (*versus* 353K)
- Mt = ~ 18min

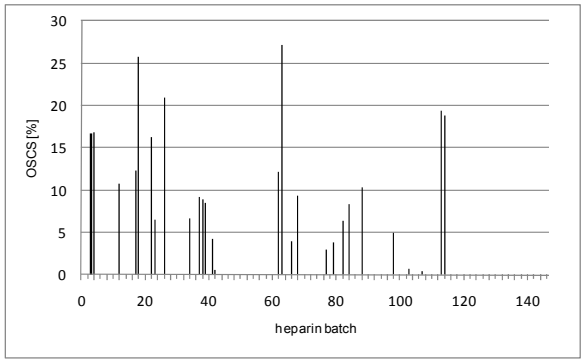
LOD = 0.1 % !



T. Beyer, B. Diehl, G. Randel, E. Humpfer, H. Schäfer, M. Spraul, C. Schollmayer, U. Holzgrabe, J. Pharm. Biomed. Anal. 48 (2008) 13-9, and Pharmeuropa Bio 2008-1, 31-39

Heparin

Analysis of ~ 150 batches in Germany
content of OSCS

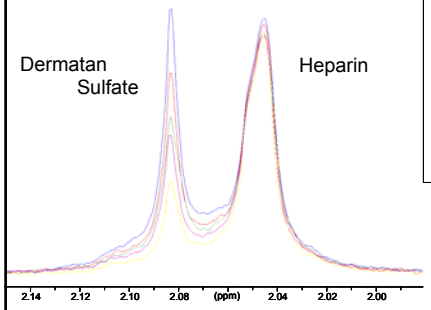


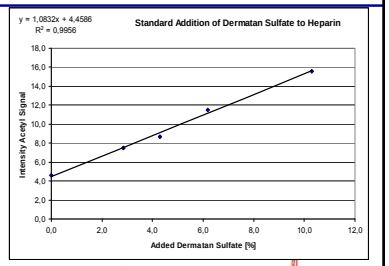
Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe, Eur. J. Pharm. Sci. 40 (2010) 297-304

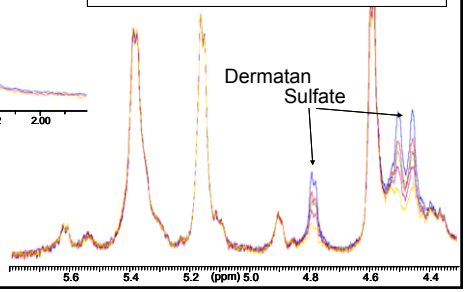
44

Evaluation of Dermatan sulfate

Standard addition of DS to heparin



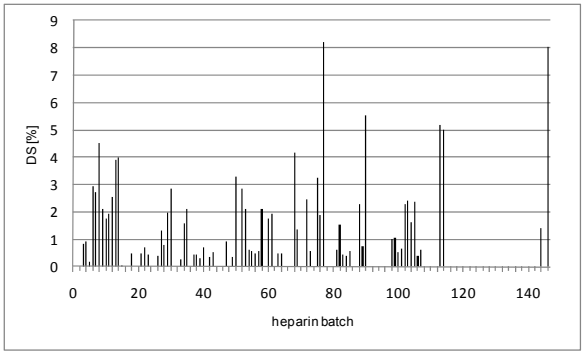




T. Beyer, B. Diehl, G. Randel, E. Humpfer, H. Schäfer, M. Spraul, C. Schollmayer, U. Holzgrabe, J. Pharm. Biomed. Anal. 48 (2008) 13-9

Heparin

Analysis of ~ 150 batches in Germany content of dermatan sulfate



Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe, Eur. J. Pharm. Sci. 40 (2010) 297-304

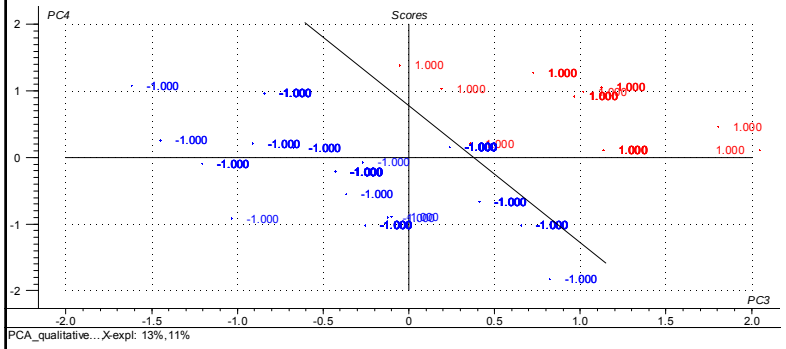
46

Heparin – PCA analysis

Analysis of ~ 150 batches in Germany

Score plot of PCA

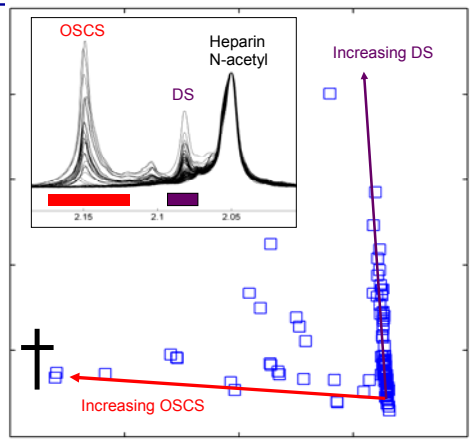
(presence (> 1%) of OSCS +1, absence of OSCS -1)



Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe, Eur. J. Pharm. Sci. 40 (2010) 297-304

47

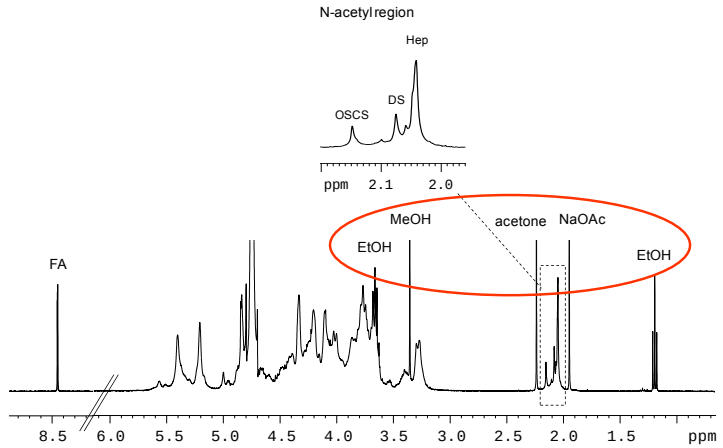
Heparin - ^{1}H NMR - PCA



Prior to bucketing spectra were aligned and scaled to the N-acetyl signal of heparin at 2.05ppm. The PC1 and PC2 scores represent 83.6% and 12.6% of the total variance in the bucket table, respectively. The PC1 scores are dominated by the effect of OSCS contamination whereas PC2 variation results from DS concentration variation.

48

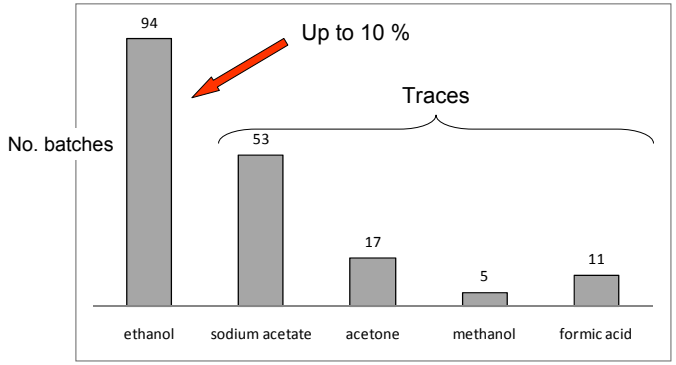
Heparin - Residual Solvents



49

Heparin

Analysis of ~ 150 batches in Germany content of residual solvents



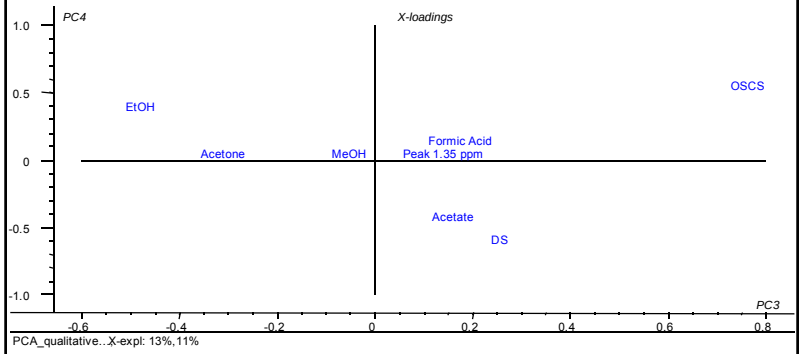
Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe, Eur. J. Pharm. Sci. 40 (2010) 297-304

50

Heparin – PCA analysis

Analysis of ~ 150 batches in Germany Correlation between all residual solvents, OSCS, DS

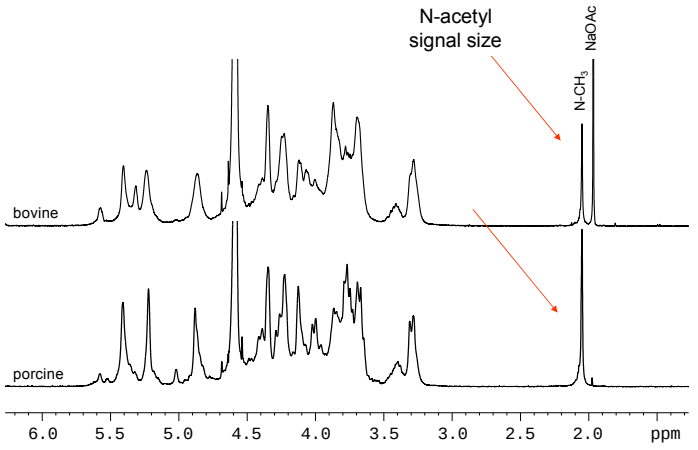
Loading plot



Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe, Eur. J. Pharm. Sci. 40 (2010) 297-304

51

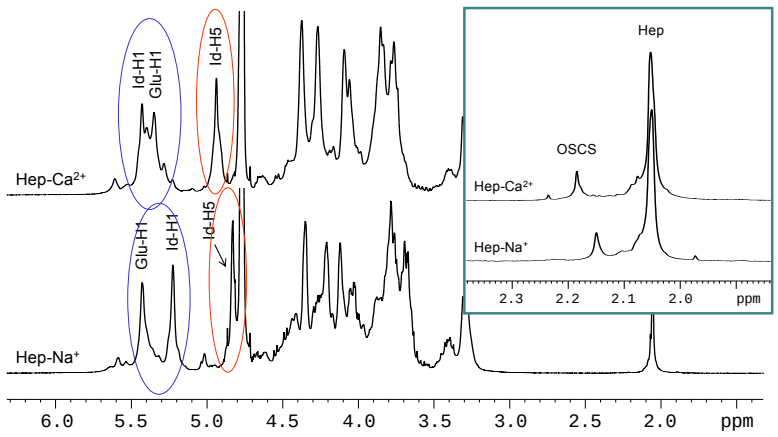
Heparin – bovine *versus* porcine



Beyer, Diehl, Holzgrabe, Bioanalytical Rev., in press

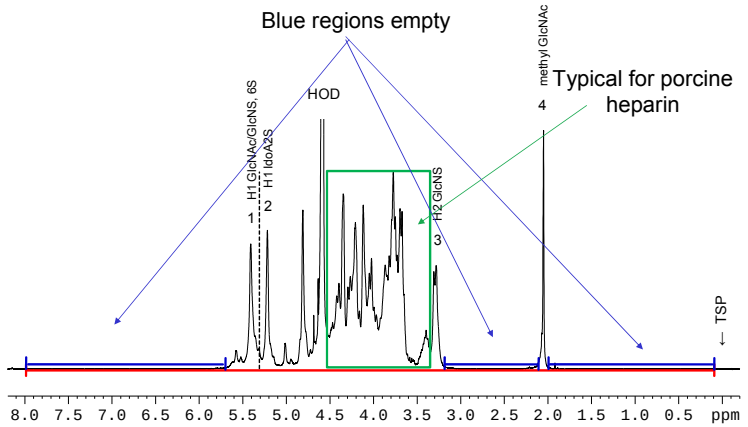
52

Heparin – Calcium *versus* Sodium



Beyer, Diehl, Holzgrabe, Bioanalytical Rev., in press

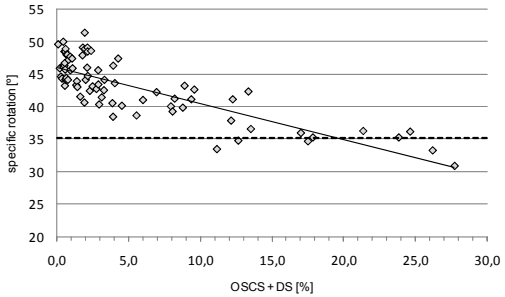
Heparin – PCA analysis



USP/PhEur: Identification test by means of NMR limits
OSCS, DS and residual solvents

Heparin

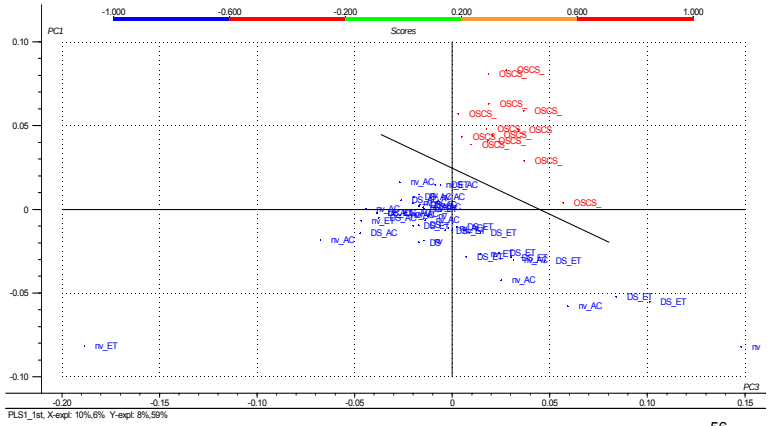
Analysis of ~ 150 batches in Germany
Optical rotation (> 35°)
PhEur 6.0



Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe,
Eur. J. Pharm. Sci. 40 (2010) 297-304

Heparin – PLS1 analysis

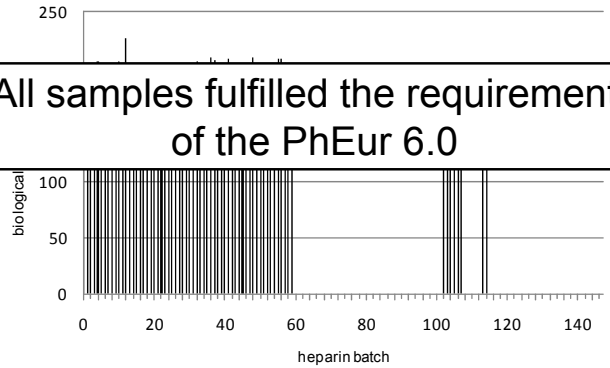
Raman data; +1 OSCS contaminated; +1 pure heparin



Norwig, Beyer, Brinz, Holzgrabe, Diller, Manns, Pharmeuropa Sci. Notes. 2009-1, 17-24.

Heparin – PCA analysis

Analysis of ~ 150 batches in Germany
Biological activity (sheep plasma clotting assay)

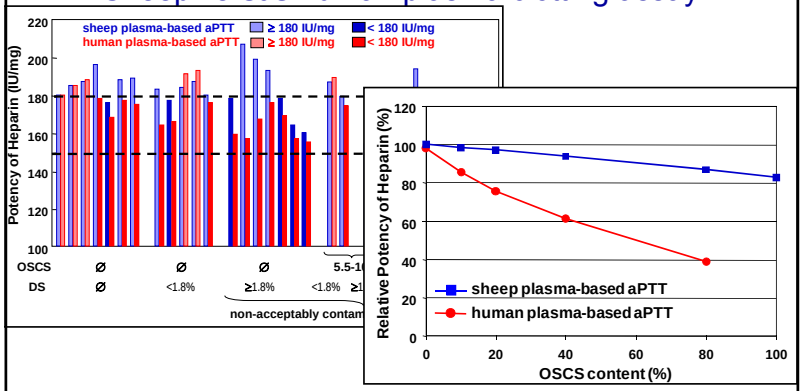


All samples fulfilled the requirements of the PhEur 6.0

Beyer, Matz, Brinz, Rädler, Wolf, Norwig, Baumann, Alban, Holzgrabe, Eur. J. Pharm. Sci. 40 (2010) 297-304

Heparin – PCA analysis

Analysis of ~ 150 batches in Germany
Sheep *versus* human plasma clotting assay



Alban, Lühn, Schiemann, Beyer, Norwig, Schilling, Rädler, Wolf, Matz, Holzgrabe, Anal. Bioanal. Chem. In press

Outline

- Fundamentals
- The gentamicin case
- Diethylene glycol in glycerin
- qNMR of Heparin: OSCS, dermatan and more
- Conclusion

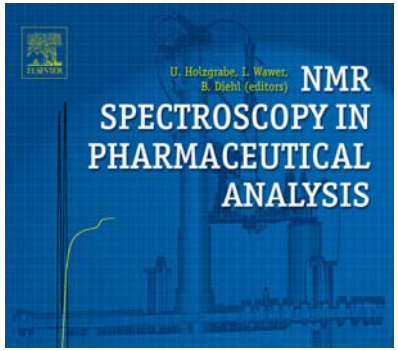
Advantages of NMR spectroscopy

- Integration of signals is more precise and accurate than HPLC analysis
- Often, no isolation of the impurity necessary
- (No) expensive chemical reference substances necessary
- Additional structural information of impurities, isomers etc.
- NMR can be quicker (no equilibration time as with HPLC)), easy to perform and more specific
  ⇒ high reproducibility

qNMR as an orthogonal method

Using NMR spectroscopy as an additional orthogonal method (to e. g. HPLC) because

- is not optimized for one synthesis pathway
- it cannot be manipulated
- gives normally more than one signal for an additional component/impurity
- deviations from a typical signature of a drug can be easily detected by simple inspection (small molecules) or statistical methods (e.g. PCA, PLS) in the case of „biologicals“
- difficult to hide an impurity



Further reading:

- Holzgrabe
  Progr. NMR spectroscopy, 57 (2010) 229-240
- Holzgrabe
  Encyclopedia of Spectroscopy and Spectrometry, Eds. J. Lindon; G. Traner, D. Koppendahl, 2nd Ed. AP 2010, Vol. 3, p. 2331-233
- Holzgrabe, Beyer
  Bioanalytical Reviews, in press

Acknowledgement

- Cowc & T.
- Bruke
- Feder and
- Feder WI), ve iF)

Thank you for attention!