肝素的来源控制、结构与活性的关系、质量趋势及市场分析

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提　纲

- 1.肝素原料来源的质量控制
- 2. 肝素的结构与生物活性的关系
- 3.未来肝素质量标准的发展趋势
- 4. 肝素全球市场简析

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1. 肝素原料来源的质量控制

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- 肝素广泛存在于哺乳动物的组织中，如肺、小肠、十二指肠、肝、心、胰脏、胎盘、血液等（目前主要应用猪来源）。
- 来源主要有猪小肠、牛肺和牛小肠、羊小肠等。

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不同来源的肝素具有不同的双糖组成

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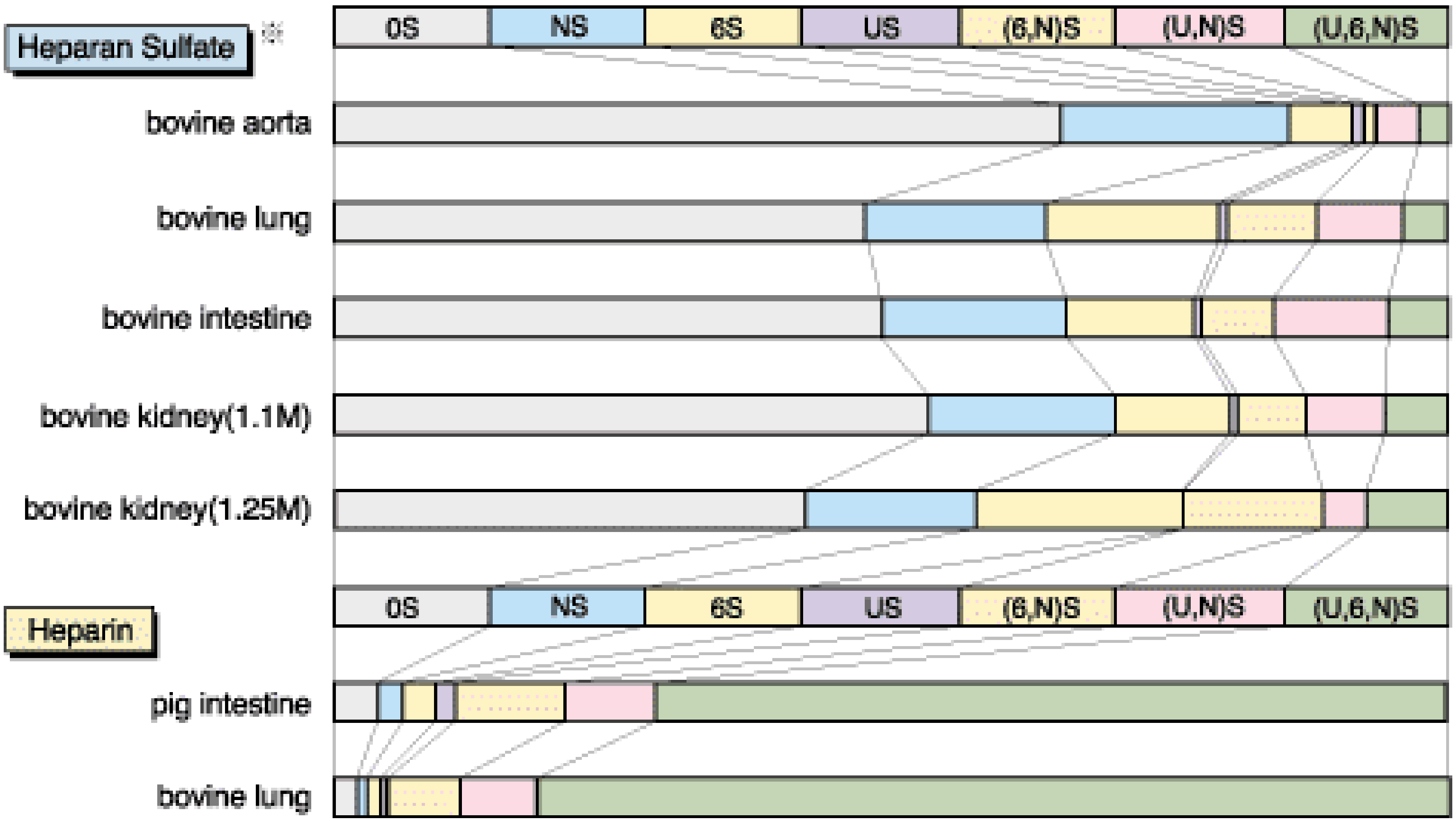

※ Maccarana, M. et al., J.Biol. Chem., 271, 17804-17810 (1996)

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1.1 生猪饲养的健康要求

- 提取肝素钠的生猪的健康状况，会直接影响到肝素钠的质量，因此必须从肝素钠的源头上，完善生猪饲养的健康要求。
- 例如在饲养过程中建立生物安全体系、减少致病性病原体的侵入，建立规范的饲养管理操作规程，合理用药、减少不必要的抗生素的使用等。

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1.2 粗品肝素钠来源的可追溯性

- 在肝素钠粗品的制备过程中，原动物组织中的蛋白质、核酸及一些其他糖胺聚糖等成分，不能被完全除尽，而这些残留的杂质常常会有种属特异性。
- 从饲养到屠宰要有严格的检验检疫报告和生猪档案管理体系，使肝素钠的来源具有可追溯性。
- 不同动物来源的肝素钠其双糖组成也会有所不同。这些都可以作为追溯粗品肝素钠来源的依据。

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1.3 粗品肝素钠来源的检测方法

- 1. Q-PCR
-    优点：灵敏、简便、快速
-    缺点：假阳性、核酸破坏难测出、无法测定精品的来源
- 2. 双糖组成测定：HPLC
-    优点：直接测定肝素，准确
-    缺点：LOD值大，水解物组成复杂，定性困难，过程复杂
- 3. ^{13}C-NMR
-    优点：直接测定肝素，准确、快速
-    缺点：LOD值大，需要特殊仪器
- 4. ELISA 法 需要测定试剂盒，目前用PAGE电泳测定，如果标记物能通过比色测定，则使用方便

1.3.1 Q-PCR

-
- 猪的引物和探针

  Sequence of forward primer
  – 5’-CATCACACTGTGTTGGTCATTGC-3’
  Sequence of reverse primer
  – 5’-CTCATGGATACCAGTCAGGTTTGT-3’
  Sequence of probe (*reverse)*
- 5’-(6-Fam)CACTGAGACACAACAGGAACTCCGCC(BHQ1)-3’

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Q-PCR

-
- 牛的引物和探针**:**

- Sequence of forward primer
  - 5’-CGGAAACGACTGAAACGACTTC-3’

  Sequence of reverse primer
  - 5’-GTGTGATCCTACCTGACTGTTCTAA-3’

  Sequence of probe (*forward)*
- 5’-(6-Fam)CAGCAGCAGCAGCAGCAGCAGCAG(BHQ1)-3’

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Q-PCR

-
- 绵羊的引物和探针:

  Sequence of forward primer
  - 5’-GATTCCTCTCGCATCCATGCAG-3’

  Sequence of reverse primer
  - 5’-GGTCCAAGTCAGCACTGGAG-3’

  Sequence of probe (*forward)*
- 5’-(6-Fam)TGGAGTCGG+**G+C**CCG(BHQ1)-3’

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Q-PCR

- 山羊的引物和探针

  Sequence of forward primer
  - 5’-GTAACGCCCTCCAAATCAATAAGA-3’

  Sequence of reverse primer
  - 5’-GGTCAACATGGCTTAGTCAAACTT-3’

  Sequence of probe (forward)
- 5’-(6-Fam)AGGAGCAGGTATCAAGCACACATCTCGT(BHQ1)-3’

Q-PCR具有很高的灵敏度

- *Working LOD bovine:* 7 pg per mg of crude heparin
- *Working LOD ovine:* 70 pg per mg of crude heparin
- *Working LOD caprine:* 4 ng per mg of crude heparin

1.3.2 SAX-HPLC测定双糖组成

- 1. 用肝素酶I、II、III将肝素降解
- 2. 用SAX-HPLC测定双糖含量组成，232nm检测

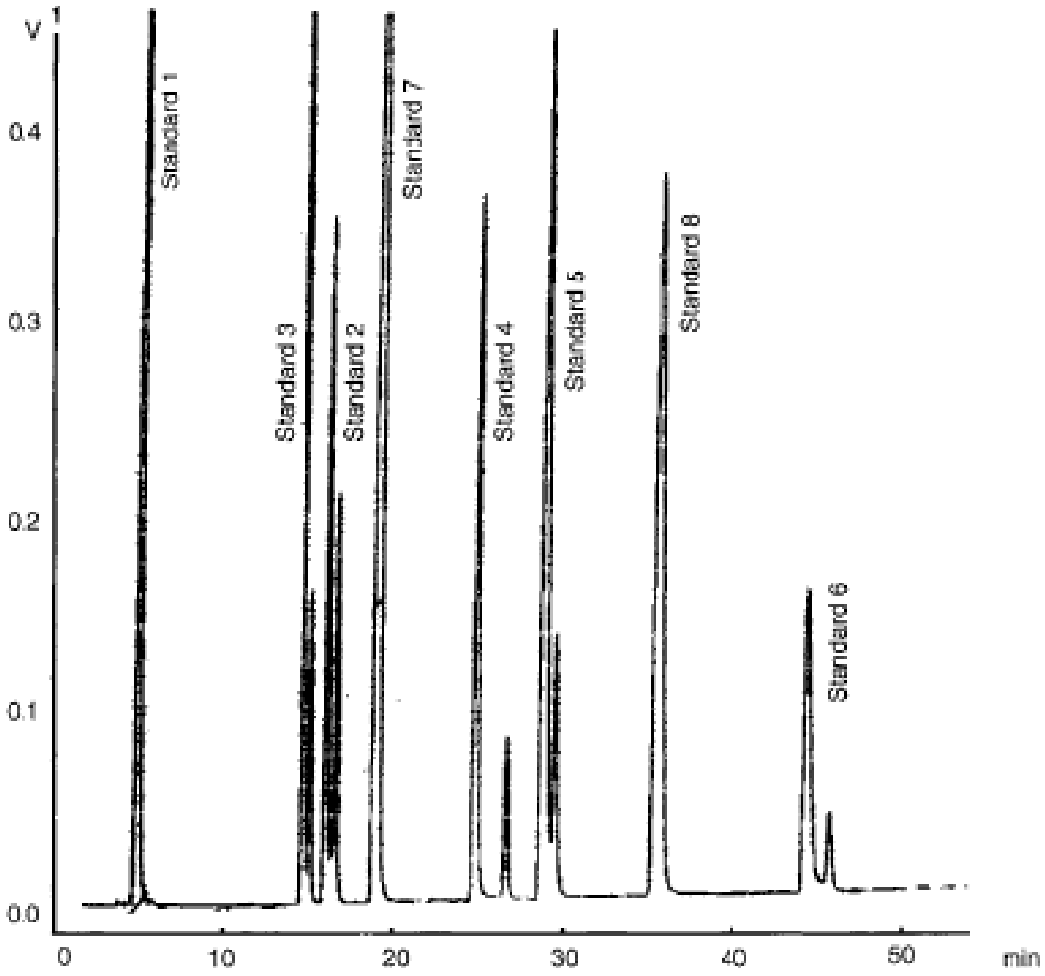

Figure 3 Disaccharide profile for eight common naturally occurring disaccharides derived from heparan sulphate. Separation was achieved using high-performance strong anion exchange chromatography (HPSAX) on a cetyl-triethylammonium ion (CTA) derivatized C18 silica-based column (C18 Hypersil) with a 0–90 min, 0–2 M ammonium methane sulphonate linear gradient, pH 3.5; flow rate 0.22 ml/min. Peaks and structures as defined in Fig. 2.

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Table 1. Disaccharide analysis of porcine, ovine and bovine heparins and their low molecular weight products(%w/w)

	ΔDiH-OS	ΔDiH-NS	ΔDiH-6S	ΔDiH-(6,N)S	ΔDiH-(U2,N)S	ΔDiH-(U2,N,6)S	Unknown (R. time 37.6 min)
Porcine							
Native	2.4	1.4	3.2	10.7	5.5	63.0	
LMW (HNO_2)	2.5	0.8	2.8	6.1	3.2	42.5	8.6
Fragmin® (HNO_2)	1.0	0.6	2.6	6.8	2.7	47.0	16.6
LMW (Cu^{2+})	1.2	0.7	2.3	8.0	3.2	49.4	
Ovine							
Native	1.1	0.3	1.0	7.5	2.0	60.0	
LMW (HNO_2)	1.5	0.5	1.0	4.2	1.2	39.4	7.2
LMW (Cu^{2+})	0.7	0.2	1.0	6.6	1.7	54.9	
Bovine							
Native	2.1	1.3	0.9	7.7	12.5	64.2	
LMW (HNO_2)	2.9	0.5	0.8	2.7	5.5	31.3	11.1
LMW (Cu^{2+})	1.1	0.6	0.8	5.3	7.1	46.5	

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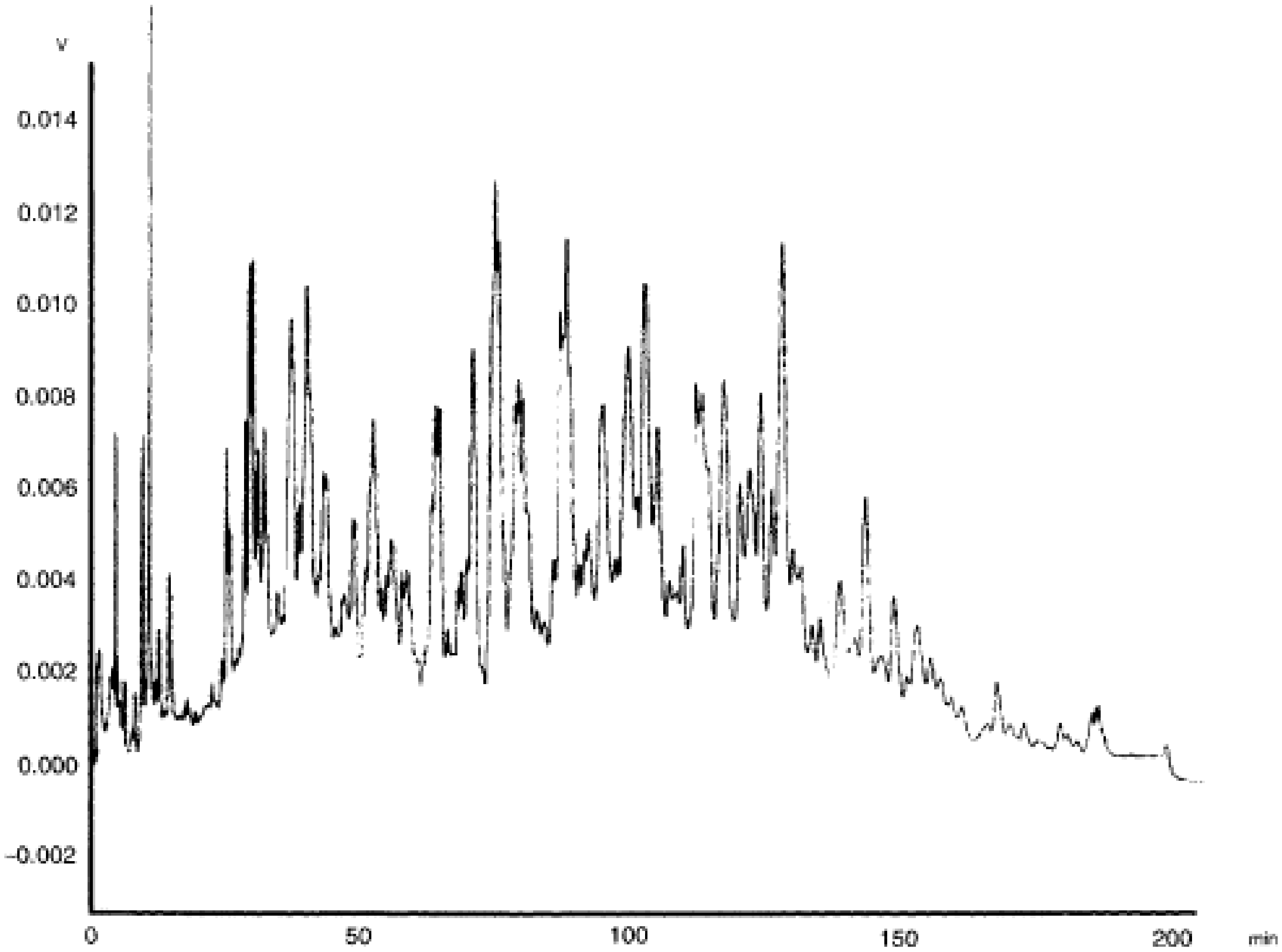

Figure 4 High-performance strong anion exchange (HPSAX) elution profile of size separated porcine mucosal derived heparan sulfate (PMHS) oligosaccharides of uniform size consisting of five disaccharide units. Run conditions were Dionex Propac PA1 column with a 0–180 min, 0–2 M NaCl linear gradient, pH 3.5; flow rate 1 ml/min.

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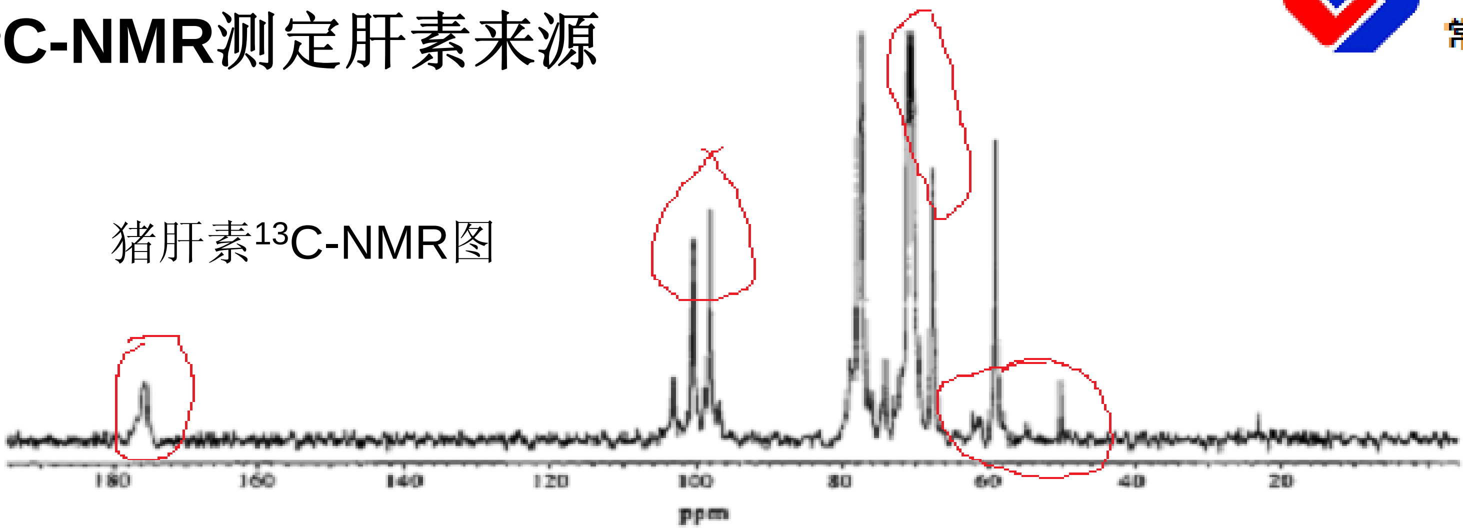
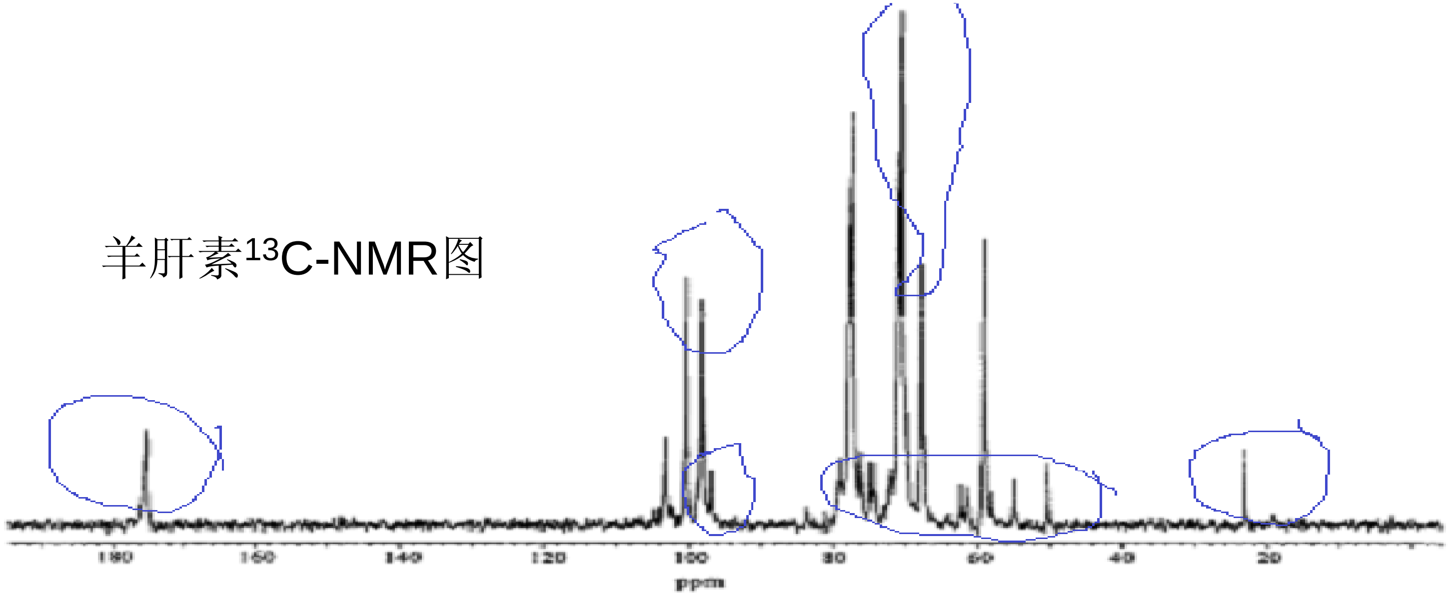
1.3.3 ^{13}C-NMR测定肝素来源



猪肝素^{13}C-NMR图



羊肝素^{13}C-NMR图

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^{13}C-NMR鉴别肝素来源

牛

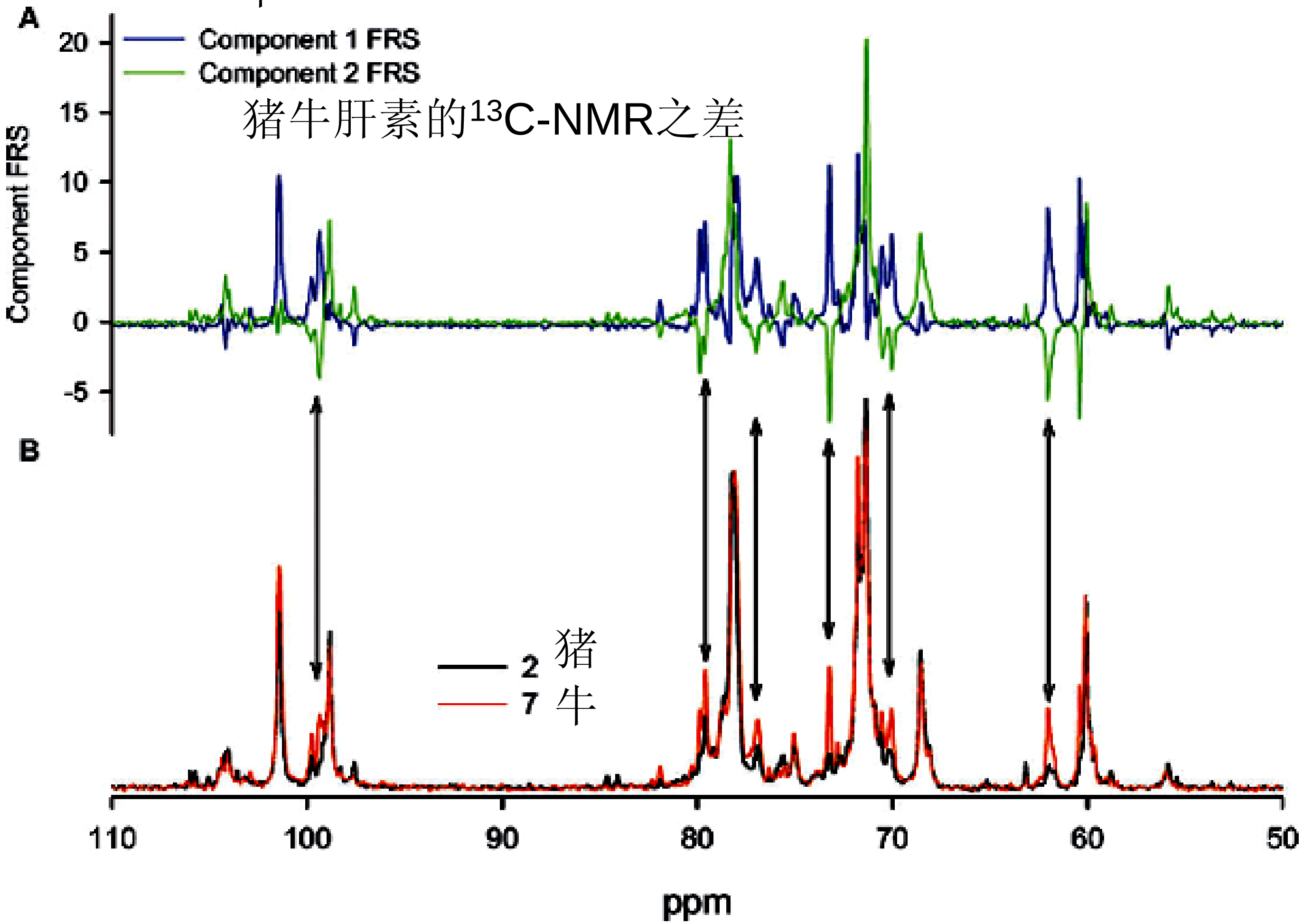

Fig. 2. (A) Component 1 and 2 factor regression score (FRS) plots, which reproduced features of the bovine spectra and the differences between the porcine and bovine spectra, respectively. (B) ^{13}C NMR spectra (as examples) of **2** (of porcine origin) and **7** (of bovine origin). Peaks picked from the FRS plots were tabulated and tentative assignments made (Table II).

FRS：因子回归积分

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1.3.4 ELISA测定

- 牛粗品肝素含有Ag1抗原，它是抑肽酶-肝素复合物，对温度、pH、化学处理等都很稳定，可以作为免疫化学法检测肝素钠粗品牛来源的靶点。
- 将粗品肝素注射给家兔，产生抗体，然后利用ELISA测定是否含有牛肝素。
- 检测限5ppm
- 同样原理用于测定绵羊肝素和山羊肝素
- 检测限是6-17ppm.

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2. 肝素的结构与生物活性的关系

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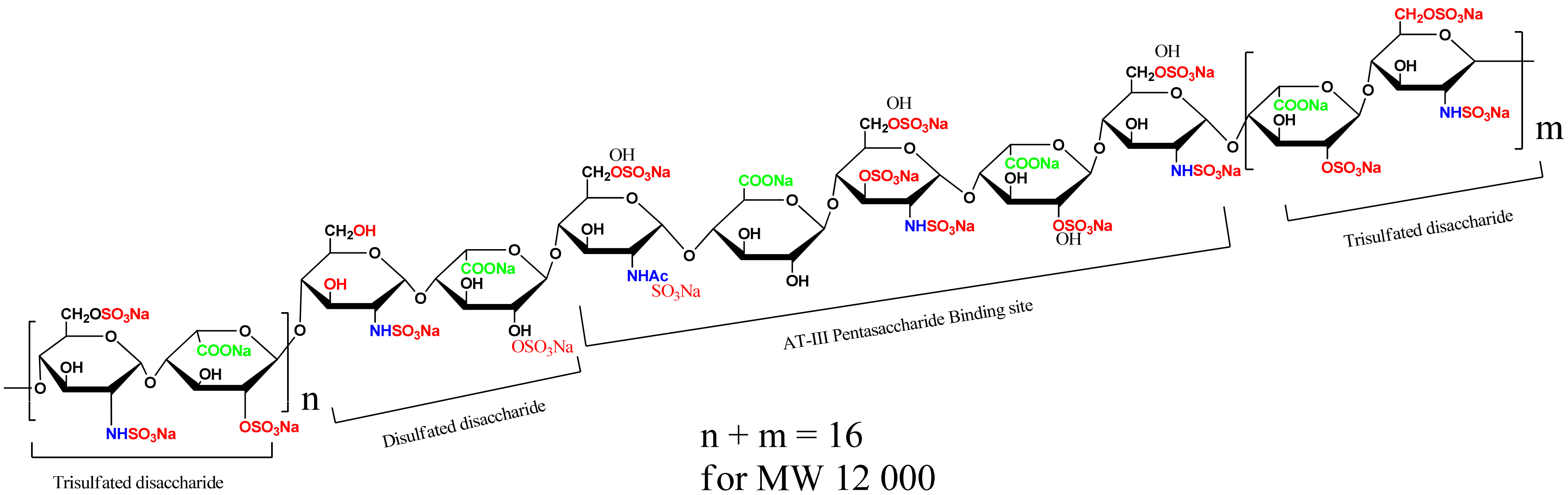
肝素的结构组成


CH2OSO3Na
OH
NHSO3Na
COONa
OH
OSO3Na
n
Trisulfated disaccharide
CH2OH
OH
NHSO3Na
COONa
OH
OH
OSO3Na
Disulfated disaccharide
OH
CH2OSO3Na
OH
NHAc
SO3Na
COONa
OH
OH
OH
CH2OSO3Na
OSO3Na
NHSO3Na
COONa
OH
OSO3Na
OH
OH
CH2OSO3Na
OH
NHSO3Na
AT-III Pentasaccharide Binding site
COONa
OH
OSO3Na
CH2OSO3Na
OH
NHSO3Na
m
Trisulfated disaccharide
n + m = 16
for MW 12 000

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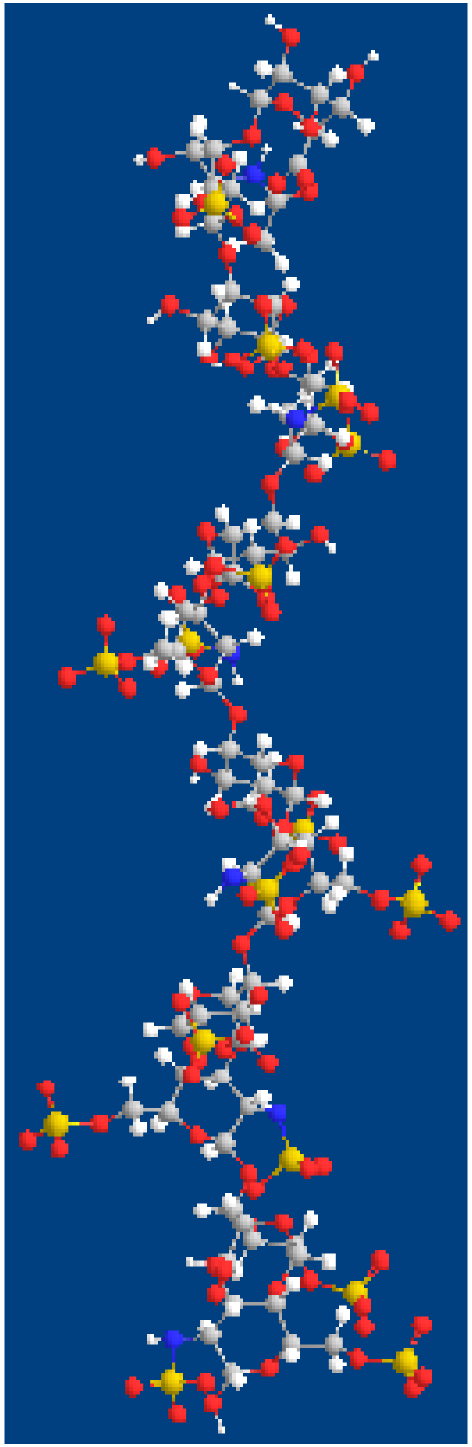
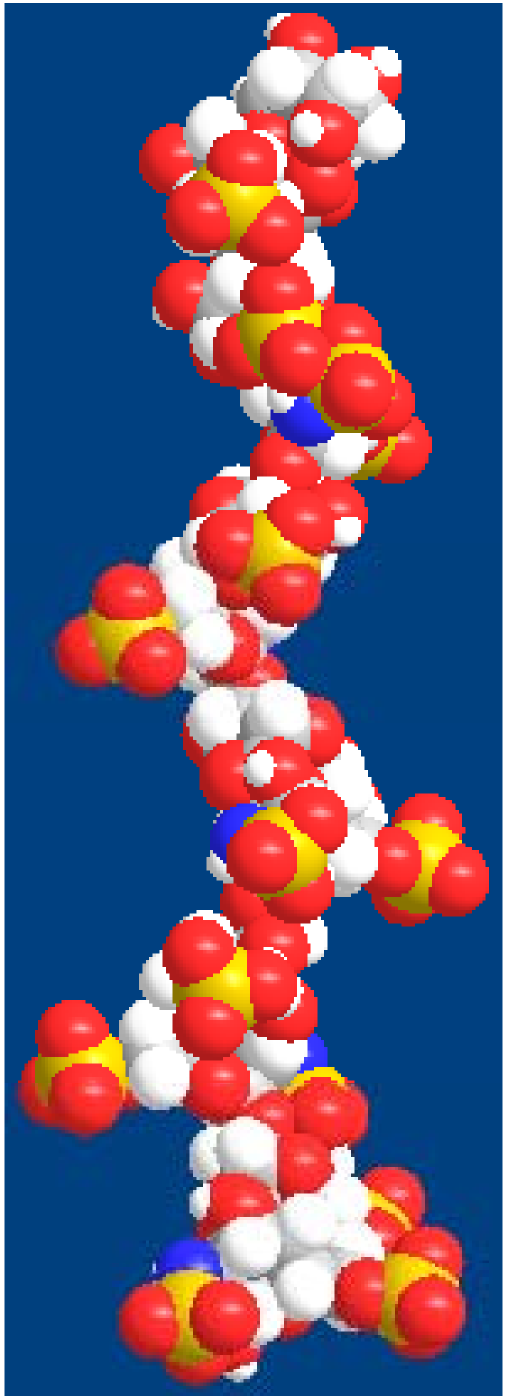
肝素十二糖结构模型





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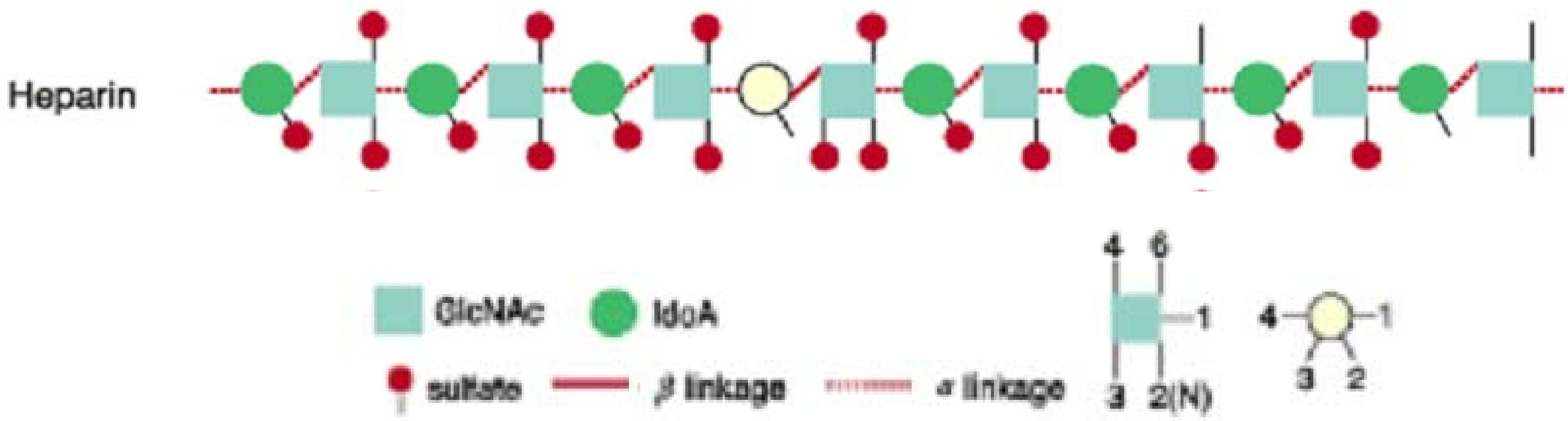
肝素分子中不同的结构片段具有不同的活性



肝素的结构示意图

(IdoA(2S)α1-4GlcNSα1-4)3 — Binding domain for FGF2

(IdoA(2S)α1-4GlcNS(6S)α1-4)3 — Binding domain for FGF1

IdoA(2S)α1-4GlcNSα1-4IdoA(2S)α1-4GlcNS(6S)a1-4IdoA(2S)a1-4GlcNSα1-4IdoA(2S)α1-4GlcNS(6S)

Binding domain for HGF

GlcNAcα1-4GlcAβ1-4GlcNS(3S)α1-4IdoA(2S)α1-4GlcNS(6S)

Binding domain for antithrombin III

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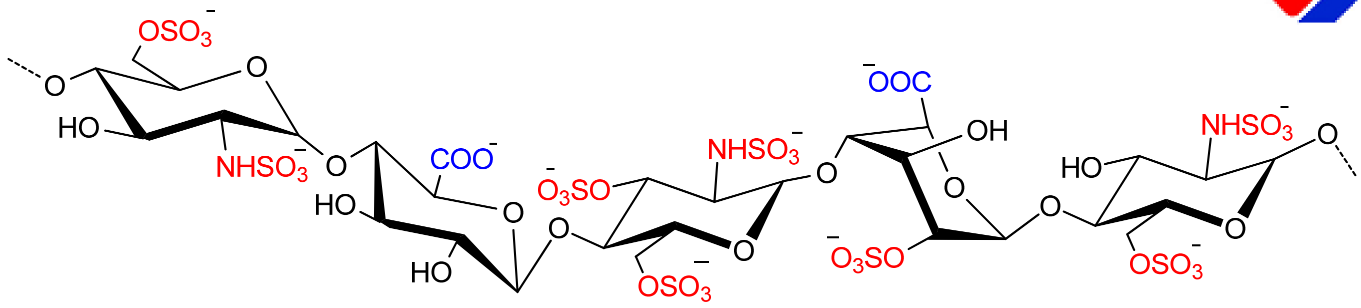
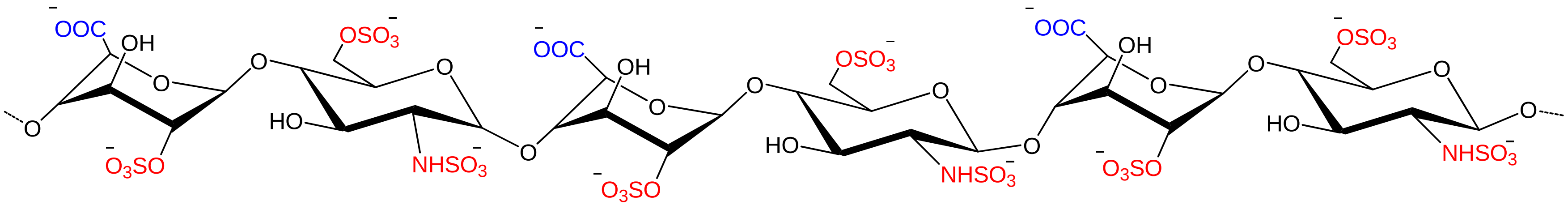
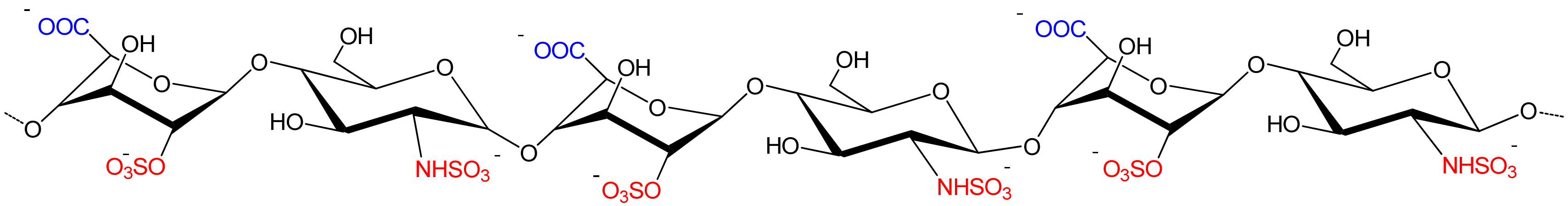
A



B



C



A：与AT III结合的结构域；B：与FGF1结合的结构域，肝素结构中的三硫酸双糖结构，占肝素结构中双糖的80 %~90 %；C：与FGF2结合的结构域；

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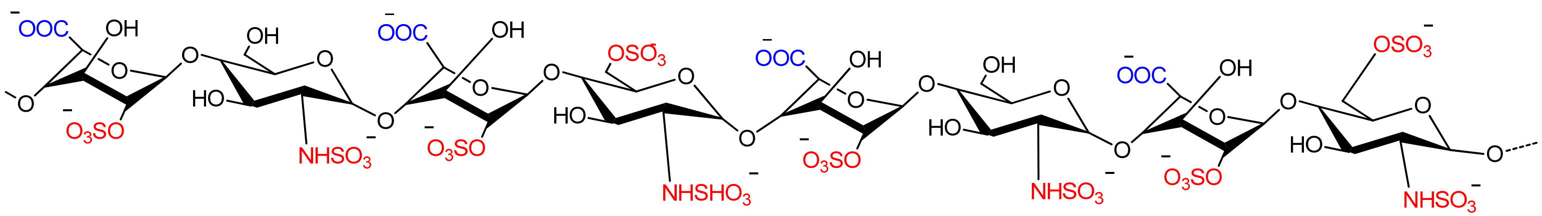
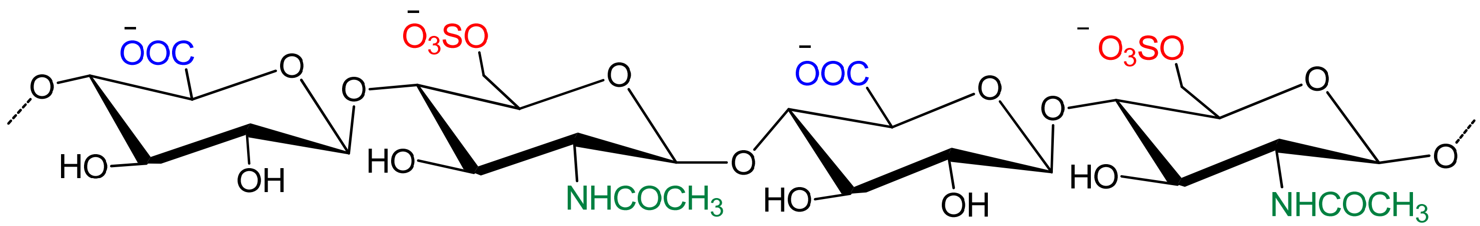
D



E



D：与HGF结合的结构域。E：单硫酸双糖结构域。

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表1 肝素结合的蛋白质及其生物活性举例

分类	肝素结合蛋白	结合后的活性
酶（**enzymes**）	凝血酶、拓扑异构酶 补体酯化酶、胞外超氧化物歧化酶	多种活性
酶抑制剂（**enzyme inhibitors** ）	**AT** Ⅲ、肝素辅因子**II**、分泌型白细胞蛋白酶抑制剂、**C1**酯酶抑制物	凝血、发炎、补体调节
细胞黏附蛋白（**cell adhesion proteins**）	**P-**选择素、**L-**选择素	细胞黏附、发炎、转移
胞外基质蛋白质（**extracellular matrix proteins**）	层粘连蛋白、纤连蛋白、胶原蛋白、凝血酶敏感蛋白、玻连蛋白、腱糖蛋白	细胞黏附、基质结构
趋化因子（**chemokines**）	血小板因子**4**、白介素	趋化作用、信号传导、发炎
生长因子（**growth factors**）	成纤维细胞生长因子、肝细胞生长因子、血管内皮生长因子	促有丝分裂、细胞迁移
形态发生素（**morphogens**）	转化生长因子**β**	细胞专一性的 组织分化、生长
酪氨酸激酶生长因子受体（**tyrosine-kinase growth factor receptors**）	转化生长因子**β**、成纤维细胞生长因子受体、血管内皮生长因子受体	促有丝分裂
脂质结合蛋白（**lipid-binding proteins**）	载脂蛋白**E**、脂蛋白脂酶、肝脂酶、钙磷脂结合蛋白类	脂质代谢、细胞膜功能
空斑蛋白（**plaque proteins**）	朊病毒蛋白质、淀粉样蛋白	空斑形成
病原体和病毒表面蛋白（**pathogen and viral surface proteins**）	疟疾环孢子蛋白、登革热病毒、单纯疱疹病毒人免疫缺陷病毒、丙型肝炎病毒	病原体感染

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3. 未来肝素质量标准的发展趋势

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3.1 USP对抗Xa/IIa活性要求

- *Anti-factor Xa* and *Anti-factor IIa* assays, with their associated variation, to show that they support the *Anti-factor Xa to Anti-factor IIa ratio* of 0.9-1.1.
- *Anti-factor IIa is not less than 180 U/mg.*

3.2 蛋白质和核酸杂质限量要求

- *Nucleotidic impurities* specification to “Not More Than (NMT) 0.1%.”
- 酶解产物用**HPLC**测定（**APP**）
- **Q-PCR**（**Sanofi-Aventis**）
- *Protein impurities* specification to “NMT 0.10%.”
- Lowry
- BCA（bicinchoninic acid，二喹啉甲酸）

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Protein impurities specification to “NMT 0.10%.”

	Accuracy	Precision	Specificity
Company 1 Lowry	Spec . 75%-125% (0.2%, 1.0%, 1.8%)	Repeat <15% Interm. <25%	Based on Water Blank
Company 2 BCA	Spec. 80%- 120% (0.05%, 0.2%, 0.5%)	Repeat < 15% Interm. < 15%	Based on Water Blank
Company 3 Lowry	Spec. 75% - 125% (0.15%, 0.3%, 0.45%)	Interm. <15%	Based on Water Blank

3.3 增加分子量及其分布的要求

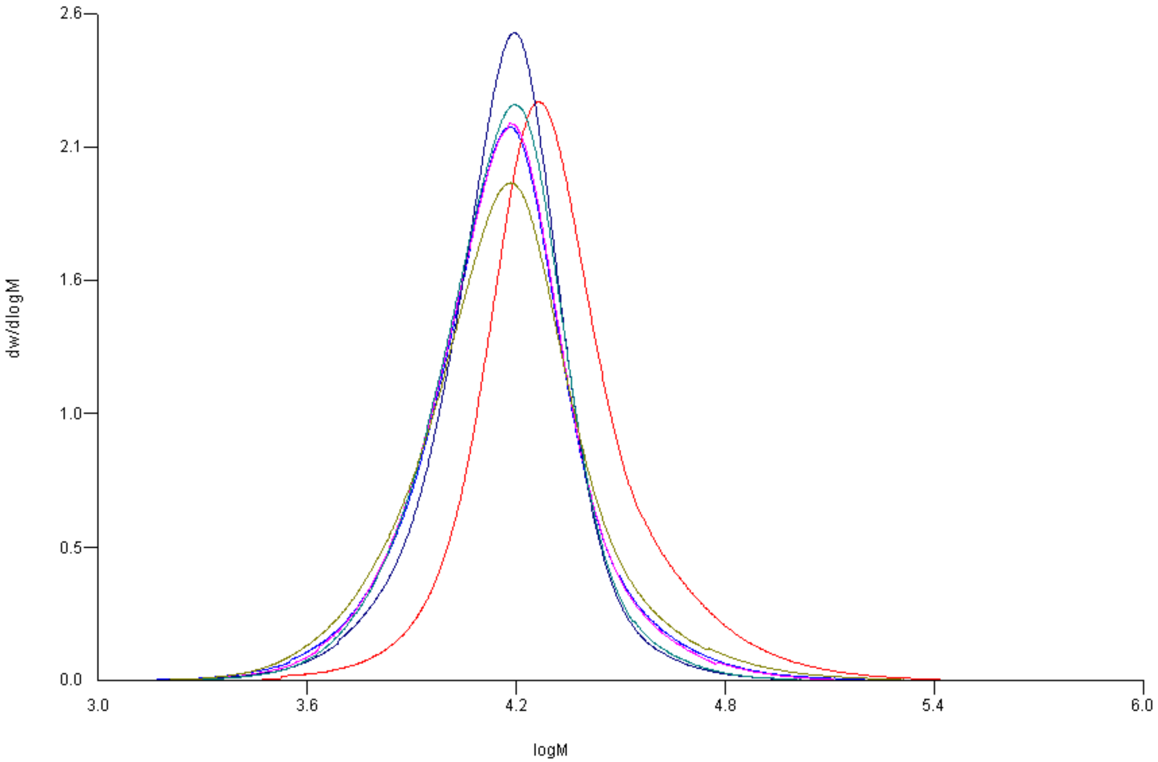
Heparin has a relatively broad molecular weight distribution.

Optimal and well controlled manufcaturing process should be able to produce batches with average molecular weight between 14,000 and 16,000 Daltons.

High molecular weight polysaccharide units are heavily sulfated and may contribute to Heparin Induced Thrombocytopenia (HIT).

Molecular weight distributions of unfractionated heparins

- Candidates for 6th IS



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UFH SEC-MALLS

UF Heparin	Mw	PolyD
Range (n=20)	14193-16232	1.01-1.04
USP	14653 ± 15	1.01 ± 0.01
Mean	**15360 ± 725 (4.7%)**	**1.03 ± 0.01 (1.0%)**

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3.4 修改 ^{1}H-NMR 方法和要求

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- Unidentified peaks have been observed in the ^{1}H NMR in the range 2.12 – 3.00 ppm. Signals at 2.17 and 2.25 ppm have been observed extensively in heparin APIs.
  - Sanofi-Aventis reported that the 2.17 ppm peak was due chiefly to 6-*O*-acetyluronic acid residues and likely result from peracetic acid （CH_3COOOH) treatment of heparin.
  - Further analytical characterization at Baxter indicates that 2.17 ppm signal has contributions from acetylation at more than one site in uronic acids due to peracetic acid treatment of heparin.
  - The 2.25 ppm signal is another *O*-acetylated species.
- Baxter has recently observed a signal at 2.15 ppm that may also be an acetylated species.
- Caution for use of plastic bottles for storage and shipment.

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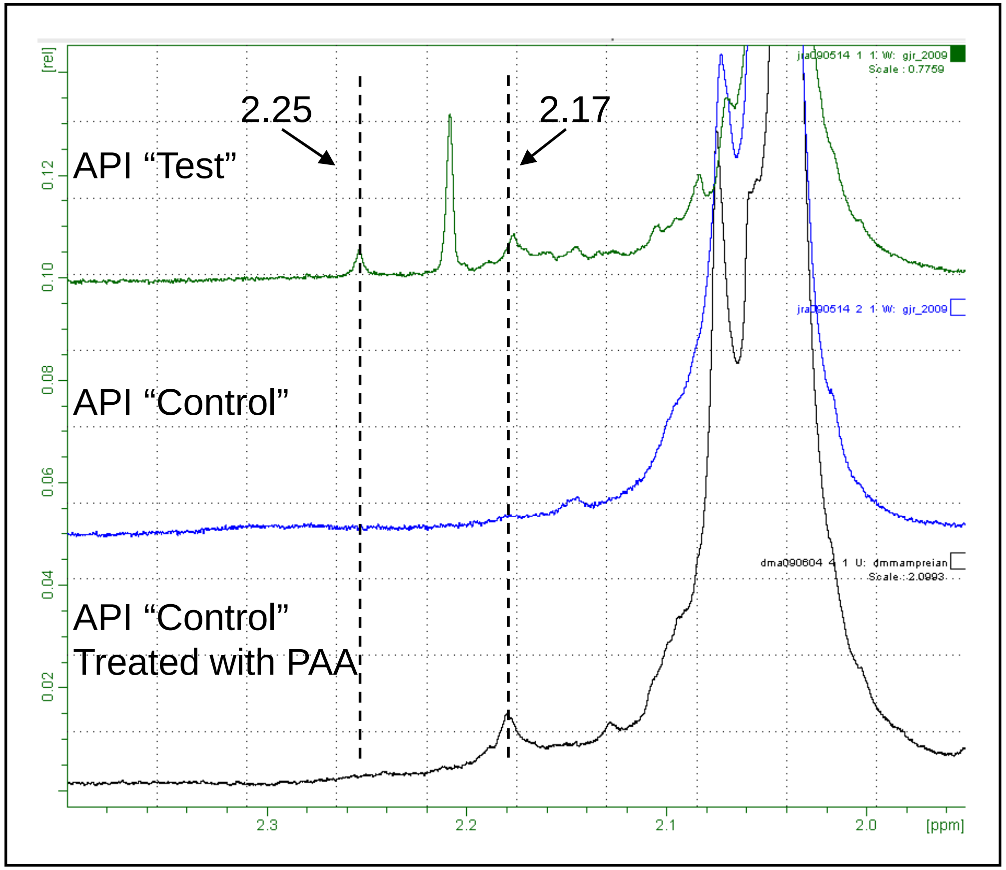
^{1}H NMR of Peracetic Acid-Treated Heparin



Conclusions: The 2.17 ppm (and sometimes the 2.25 ppm) peak can be generated from a control lot of heparin API under a variety of experimental conditions using peracetic acid.

^{1}H NMR Spectra of Control Heparin After Treatment with $^{13}C_4$-Labeled Acetic Anhydride

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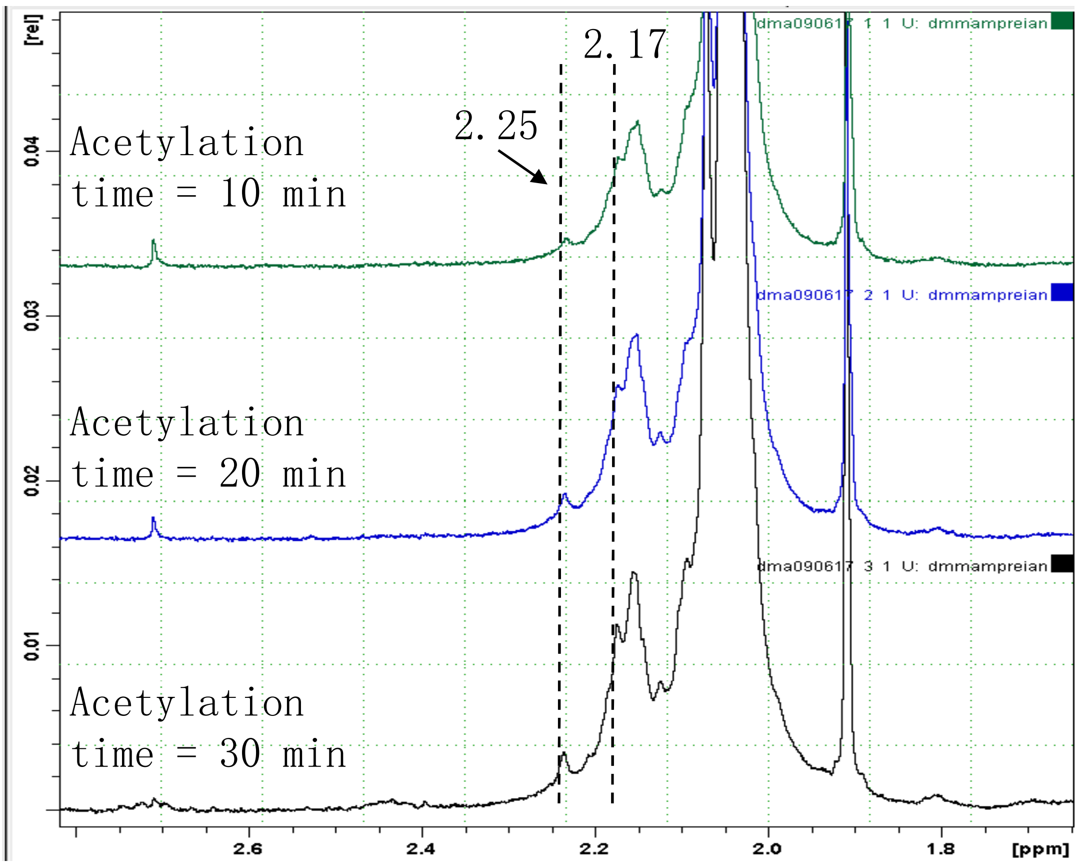

Conclusion: This data demonstrates that the 2.17 and 2.25 ppm peaks can be formed using acetic anhydride as well as peracetic acid, suggesting an acetylation rather than an oxidation.

4. 肝素的全球市场简析

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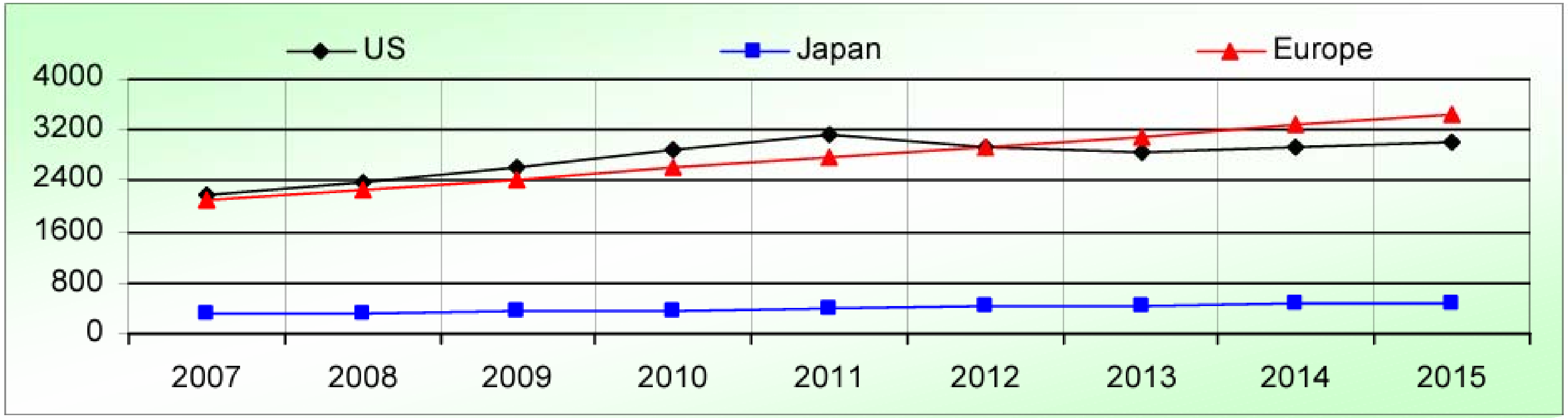
注射抗凝剂（肝素+低分子肝素）的区域份额

TABLE 5: Global Injectable Anticoagulants Market by Region for the Period 2007-2015 (Sales in US$ Million)

Region/Country	2007	2008	2009	2010	2011	2012	2013	2014	2015	% CAGR
US	2173.76	2393.64	2630.76	2886.33	3127.69	2933.04	2869.32	2936.08	3026.82	4.23
Japan	299.48	322.44	346.44	371.41	395.71	416.38	437.30	458.34	479.48	6.06
Europe	2100.04	2261.87	2428.98	2600.82	2775.91	2937.34	3101.00	3268.58	3438.78	6.36
Rest of World	407.25	436.83	467.52	498.73	529.67	558.08	586.87	616.26	645.71	5.93
Total	**4980.53**	**5414.77**	**5873.71**	**6357.29**	**6828.98**	**6844.84**	**6994.48**	**7279.27**	**7590.79**	**5.41**

2008-2009: BizAcumen Estimates
Data is reported at the Retail Level

2010-2015: BizAcumen Projections



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2007-2015年全球肝素原料药的市场需求及预测
50
45
40
35
30
25
20
15
10
5
0
14%
12%
10%
8%
6%
4%
2%
0%
-2%
23.9
23.6
23.6
26.2
29.3
32.9
35.7
39.5
44.3
0.23%
-1.25%
0%
11.02%
11.83%
12.29%
8.51%
10.64%
12.15%
2007年
2008年
2009年
2010年
2011年E
2012年E
2013年E
2014年E
2015年E
需求量：万亿单位
同比增长

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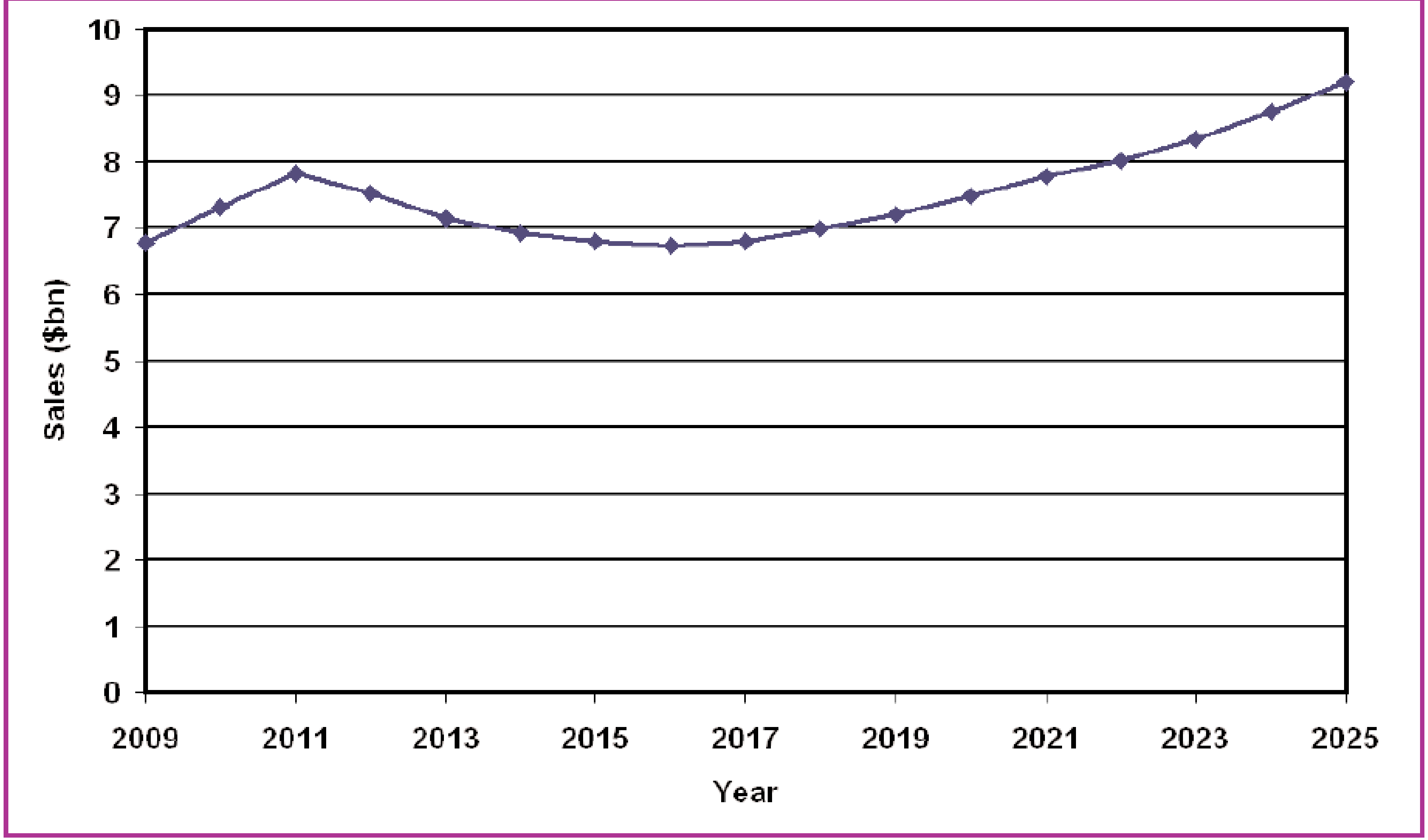
2009至2025年肝素类产品的销售收入预测

Figure 4.4 Heparins: Market Forecast ($bn), 2010-2025



Source: visiongain, 2010

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注射抗凝剂（肝素+低分子肝素）的区域比例

TABLE 6: Percentage Breakdown of Global Injectable Anticoagulants Market by Region for the Years 2008 & 2012

Region/Country	2008	2012
US	44.21	42.85
Japan	5.95	6.08
Europe	41.77	42.92
Rest of World	8.07	8.15
Total	100.00	100.00

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肝素和低分子肝素的市场趋势

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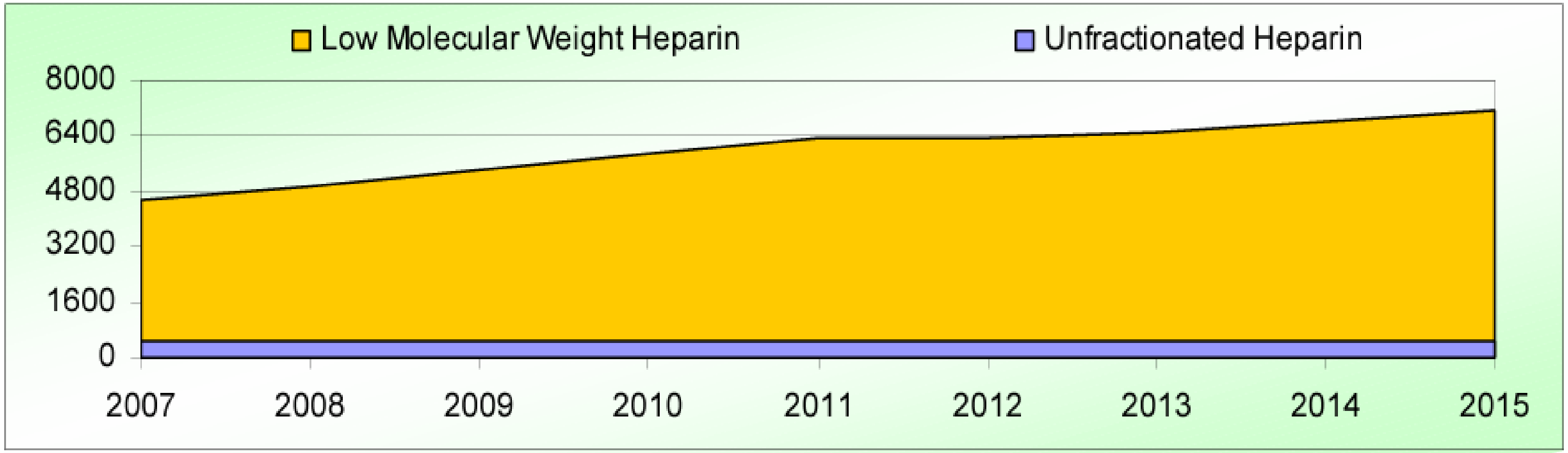
TABLE 7: Global Injectable Anticoagulants Market by Segment for the Period 2007-2015 (Sales in US$ Million)

Product Segment	2007	2008	2009	2010	2011	2012	2013	2014	2015	% CAGR
Unfractionated Heparin	469.71	477.88	481.81	481.79	475.88	462.62	452.03	443.18	435.77	-0.93
Low Molecular Weight Heparin	4510.82	4936.90	5391.90	5875.51	6353.10	6382.21	6542.46	6836.08	7155.01	5.94
Total	**4980.53**	**5414.77**	**5873.71**	**6357.29**	**6828.98**	**6844.84**	**6994.48**	**7279.27**	**7590.79**	**5.41**

2008-2009: BizAcumen Estimates
Data is reported at the Retail Level

2010-2015: BizAcumen Projections



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肝素和低分子肝素的市场比例

TABLE 8: Percentage Breakdown of Global Injectable Anticoagulants Market by Segment for the Years 2008 & 2012

Product Segment	2008	2012
Unfractionated Heparin	8.83	6.76
Low Molecular Weight Heparin	91.17	93.24
Total	100.00	100.00

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未分级肝素的区域份额

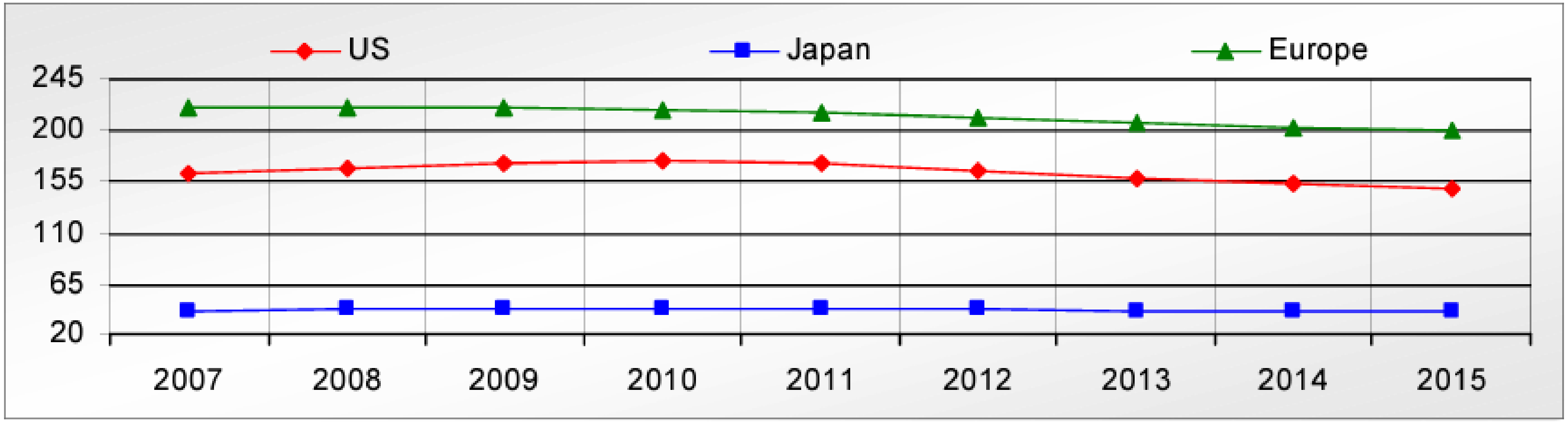
TABLE 9: Global Unfractionated Heparin Market by Region for the Period 2007-2015 (Sales in US$ Million)

Region/Country	2007	2008	2009	2010	2011	2012	2013	2014	2015	% CAGR
US	162.2	167.5	170.8	173.3	171.6	163.7	158.4	153.9	150.0	-0.97
Japan	40.6	41.3	41.7	42.0	41.8	41.2	40.5	40.0	39.5	-0.33
Europe	220.1	221.6	221.3	218.6	215.2	211.0	207.0	203.7	201.1	-1.13
Rest of World	46.8	47.5	48.0	47.8	47.3	46.7	46.1	45.6	45.1	-0.45
Total	**469.7**	**477.9**	**481.8**	**481.8**	**475.9**	**462.6**	**452.0**	**443.2**	**435.8**	**-0.93**

2008-2009: BizAcumen Estimates

2010-2015: BizAcumen Projections

Data is reported at the Retail Level



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未分级肝素的区域比例

TABLE 10: Percentage Breakdown of Global Unfractionated Heparin Market by Region for the Years 2008 & 2012

Region/Country	2008	2012
US	35.04	35.39
Japan	8.64	8.91
Europe	46.38	45.61
Rest of World	9.94	10.09
Total	100.00	100.00

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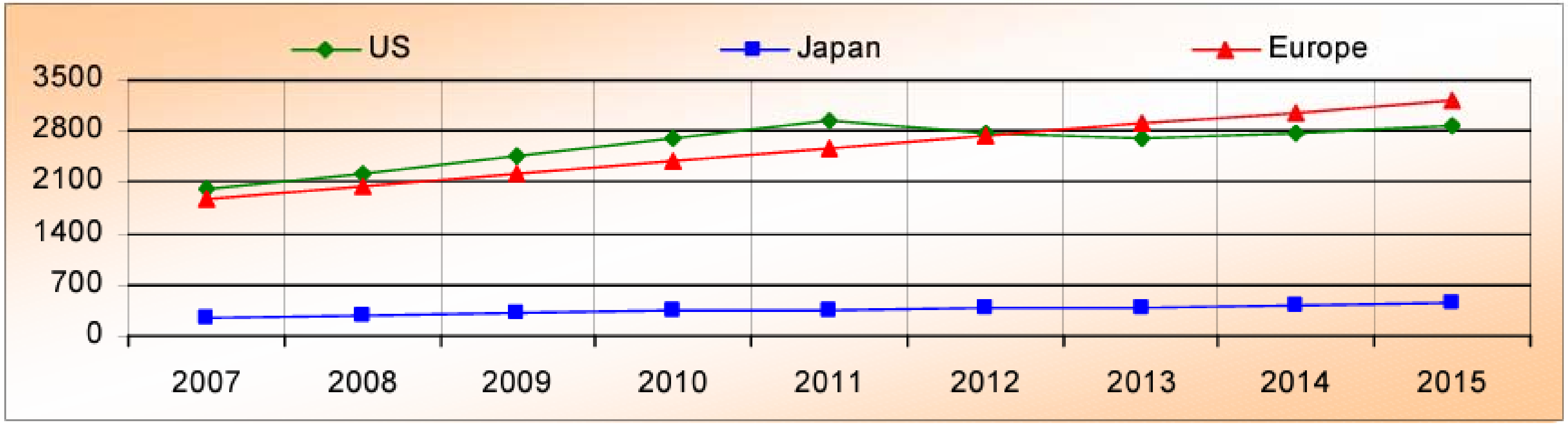
低分子肝素的区域份额

TABLE 11: Global Low Molecular Weight Heparin Market by Region for the Period 2007-2015 (Sales in US$ Million)

Region/Country	2007	2008	2009	2010	2011	2012	2013	2014	2015	% CAGR
US	2011.55	2226.17	2459.92	2713.05	2956.14	2769.31	2710.88	2782.18	2876.77	4.57
Japan	258.91	281.16	304.74	329.37	353.90	375.18	396.79	418.37	439.96	6.85
Europe	1879.89	2040.22	2207.67	2382.19	2560.72	2726.30	2894.02	3064.86	3237.70	7.03
Rest of World	360.47	389.35	419.56	450.90	482.33	511.42	540.78	570.68	600.59	6.59
Total	**4510.82**	**4936.90**	**5391.90**	**5875.51**	**6353.10**	**6382.21**	**6542.46**	**6836.08**	**7155.01**	**5.94**

2008-2009: BizAcumen Estimates
Data is reported at the Retail Level

2010-2015: BizAcumen Projections



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低分子肝素的区域比例

TABLE 12: Percentage Breakdown of Global Low Molecular Weight Heparin Market by Region for the Years 2008 & 2012

Region/Country	2008	2012
US	45.09	43.39
Japan	5.69	5.88
Europe	41.33	42.72
Rest of World	7.89	8.01
Total	100.00	100.00

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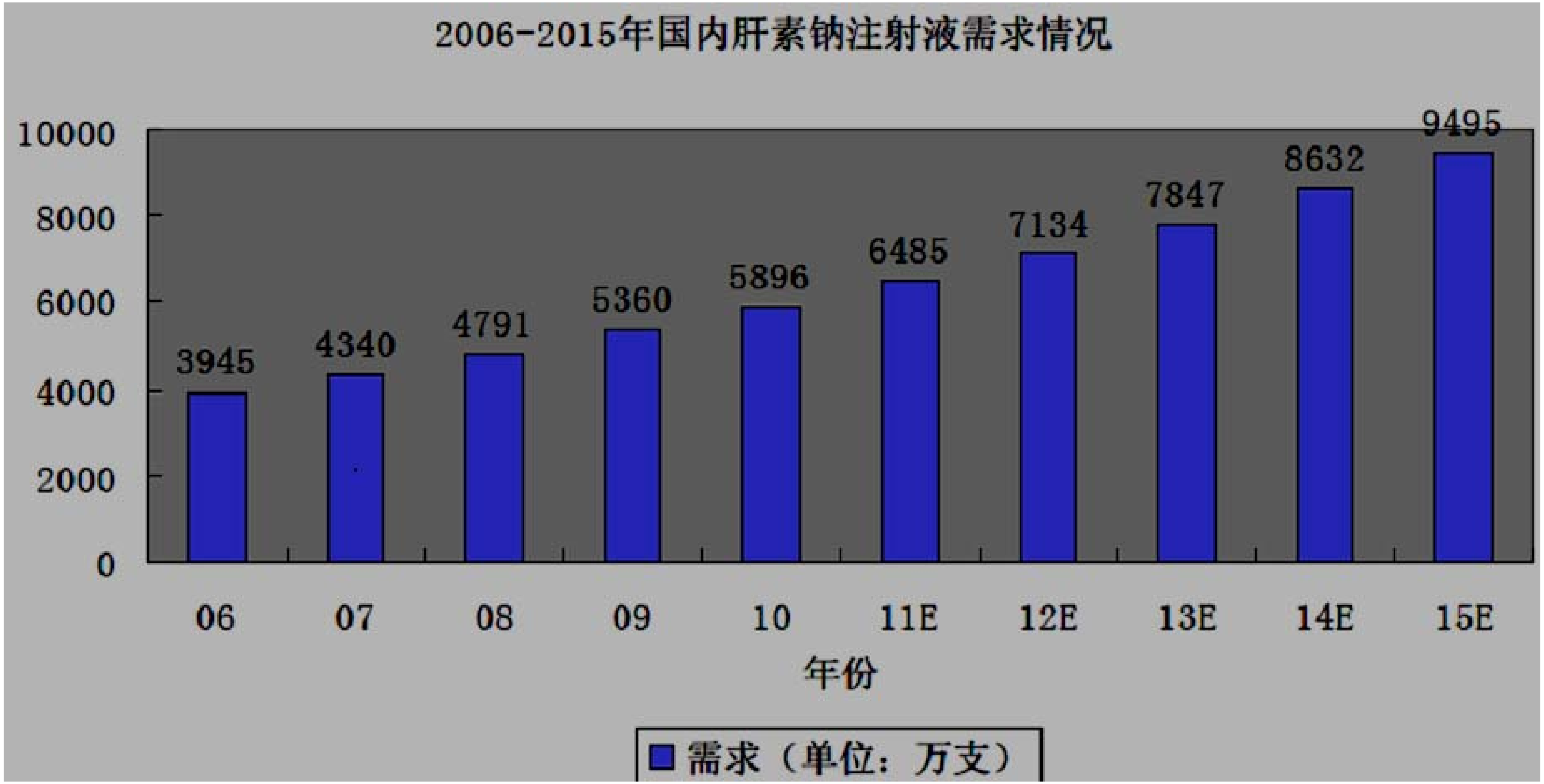
国内肝素注射剂的需求量



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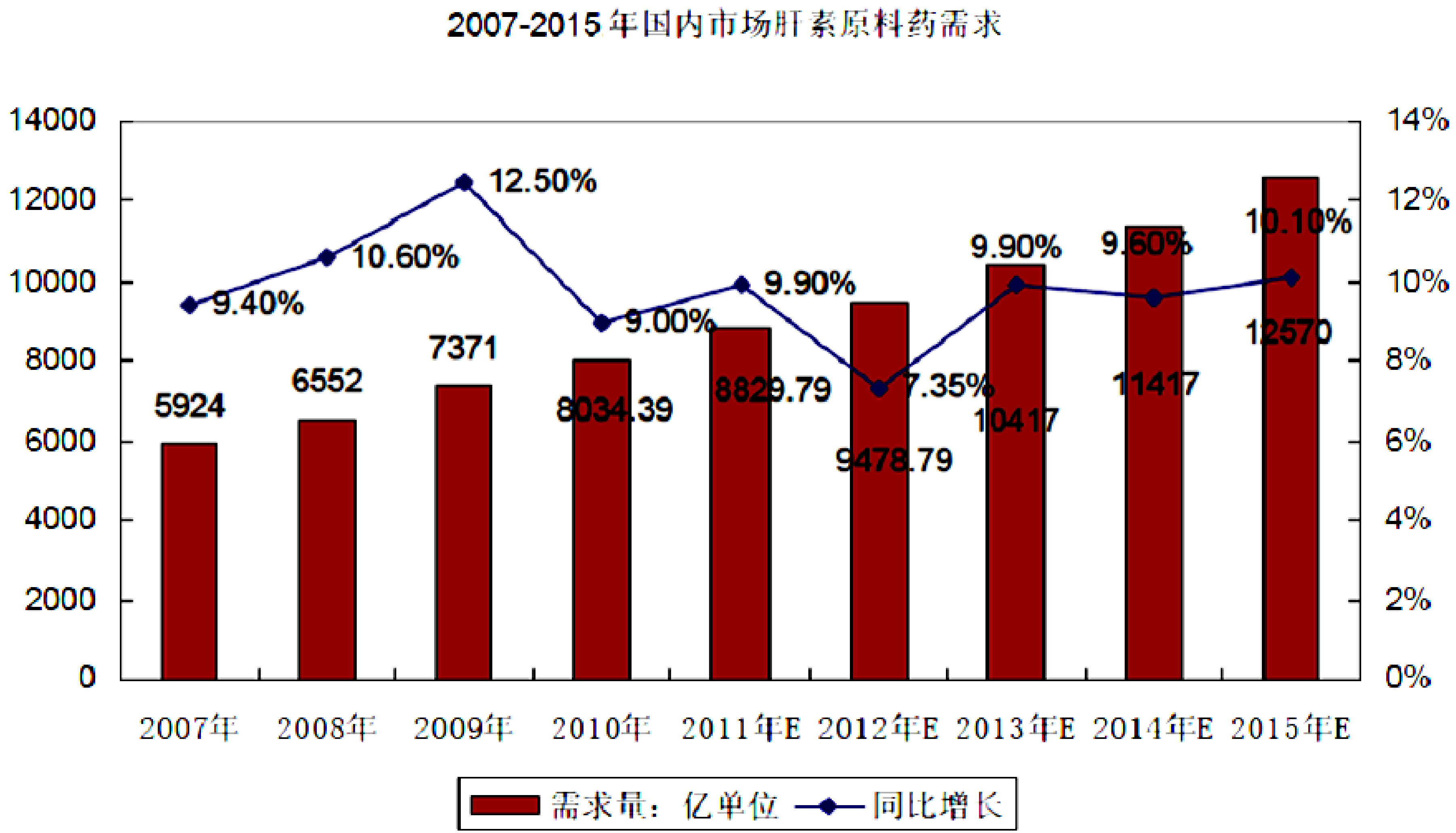
2007-2015年国内市场肝素原料药需求



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中国肝素出口所占全球比例

	2007	2008	2009	2010	2011E	2012E	复合增长率
全球用量万亿单位）	**23.9**	**23.6**	**23.6**	**26.2**	**29.3**	**32.9**	
中国出口量（万亿单位）	**11.9**	**8.5**	**13.4**	**15.9**	**18.9**	**22.4**	6.12%
中国占比（%）	**49.79**	**36.02**	**56.78**	**60.69**	**64.51**	**68.08**	

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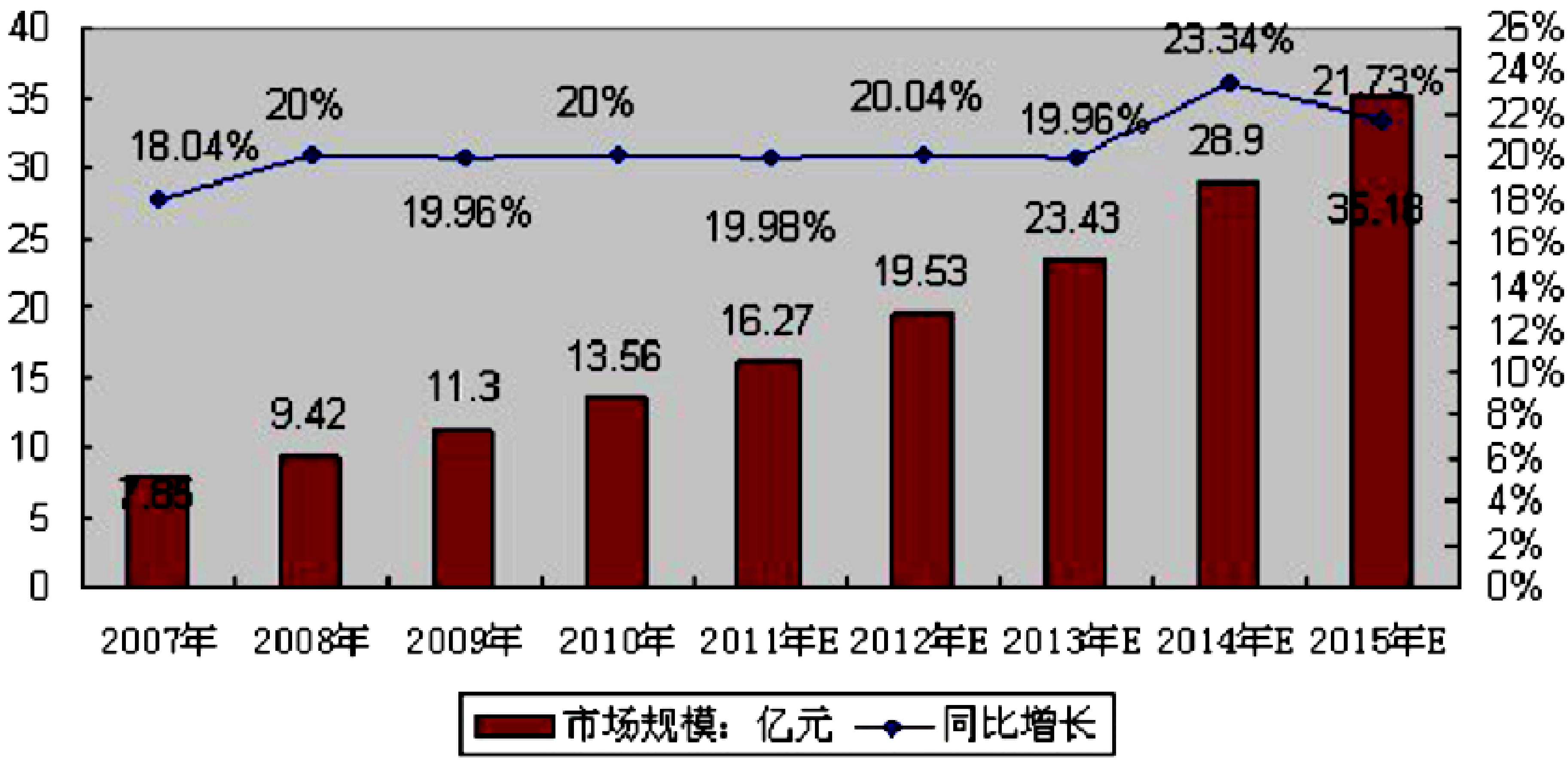
2007-2015年国内低分子肝素药物市场规模及走势



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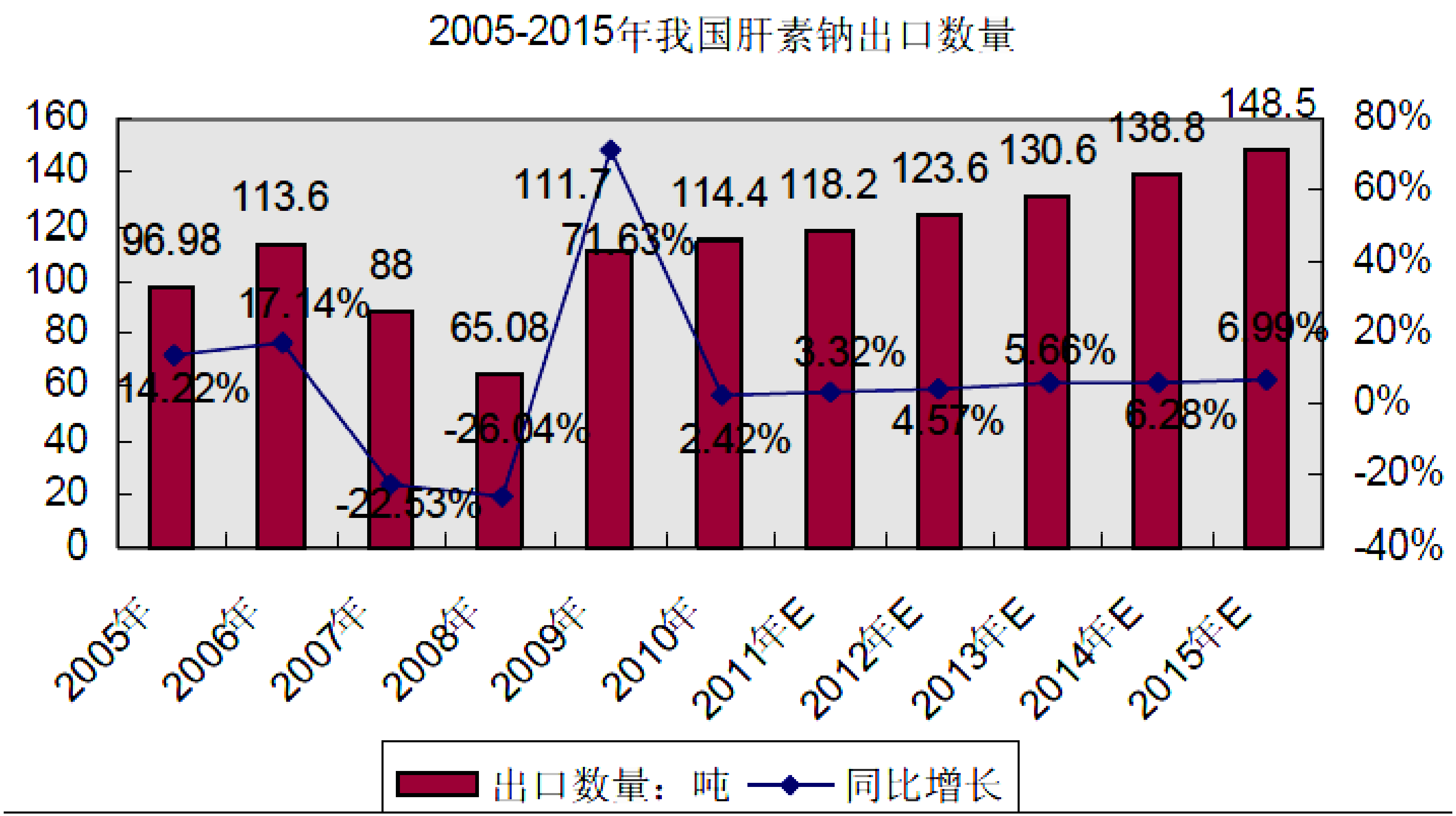
2005-2015年我国肝素钠出口数量
160
140
120
100
80
60
40
20
0
80%
60%
40%
20%
0%
-20%
-40%
96.98
113.6
88
65.08
111.7
114.4
118.2
123.6
130.6
138.8
148.5
14.22%
17.14%
-22.53%
-26.04%
71.63%
2.42%
3.32%
4.57%
5.66%
6.28%
6.99%
2005年
2006年
2007年
2008年
2009年
2010年
2011年E
2012年E
2013年E
2014年E
2015年E
出口数量：吨
同比增长

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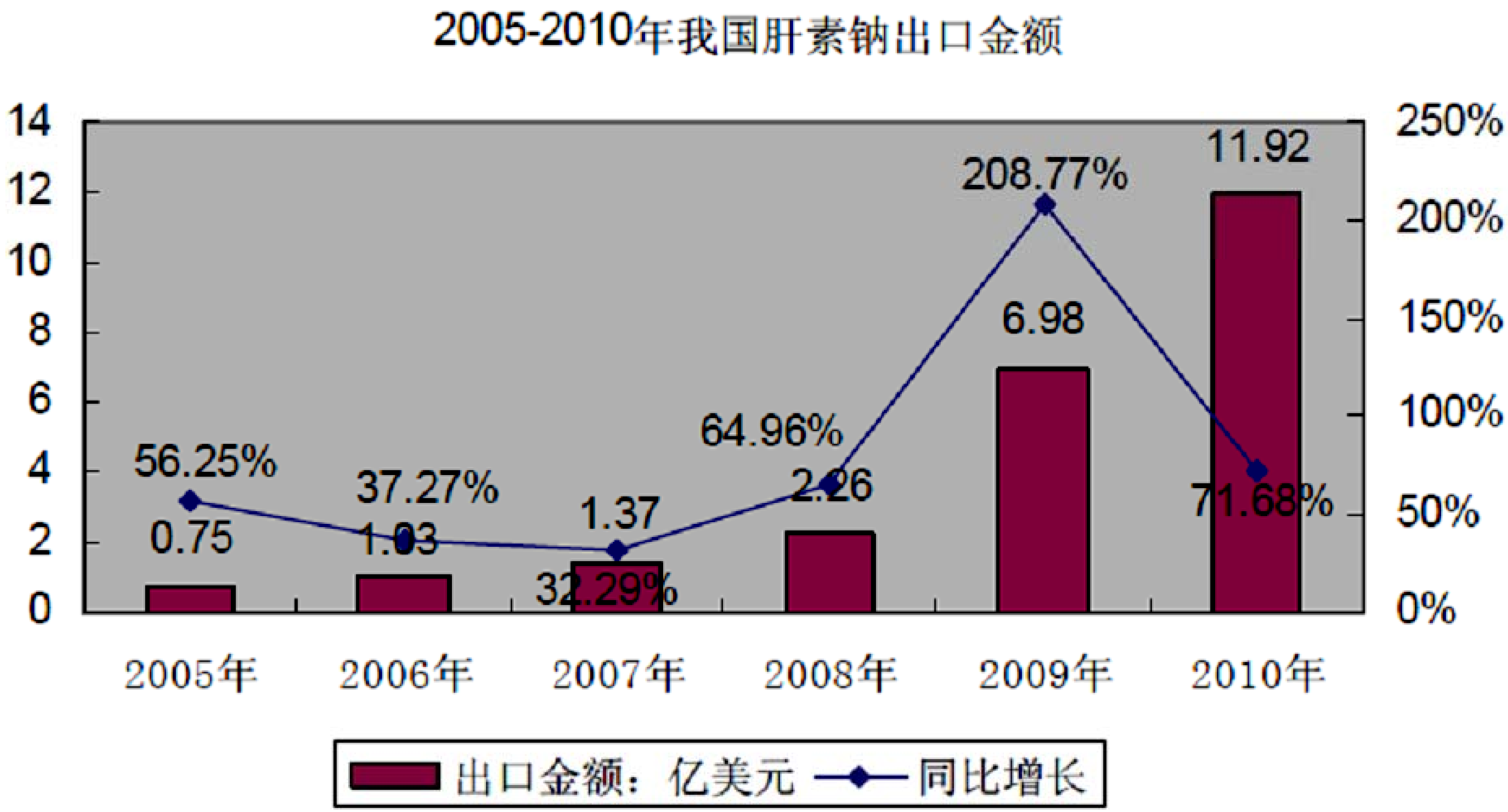
2005-2010年我国肝素钠出口金额
14
12
10
8
6
4
2
0
250%
200%
150%
100%
50%
0%
56.25%
0.75
37.27%
1.03
1.37
32.29%
64.96%
2.26
208.77%
6.98
11.92
71.68%
2005年
2006年
2007年
2008年
2009年
2010年
出口金额：亿美元
同比增长

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THANK YOU

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