Comparative Neutralization of Lung- and Mucosal-Derived Heparin by Protamine Sulfate Using *In Vitro* and *In Vivo* Methods

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Abstract □ The objective of this investigation was to compare the number of heparin units neutralized by protamine sulfate as a function of heparin source, potency, and manufacturing process. Two experiments were conducted. In the first, results obtained by the *in vivo* response in rabbits were shown to agree with results obtained by the USP *in vitro* assay. The second compared the neutralization of several samples of sodium heparin by the USP *in vitro* procedure according to the parameters outlined. Results reported establish significant neutralization differences.

Keyphrases □ Heparin, lung, mucosal derived—comparison of neutralization by protamine sulfate, *in vivo*, *in vitro* □ Protamine sulfate, neutralization of heparin—comparison, lung, mucosal derived, *in vivo*, *in vitro* □ Potency, heparin—correlation with neutralization values

In recent years, some clinicians have expressed concern over the apparent variability in patients of the neutralization of sodium heparin by protamine sulfate. Walton *et al.* (1) reported that the number of heparin units neutralized by protamine sulfate varies with the source and specific activity of the sodium heparin.

Results similar to those reported by Walton *et al.* (1) were observed in evaluating heparin samples derived respectively from lung and mucosa, currently the primary market sources. Sodium heparin derived from different source materials was neutralized by protamine sulfate according to the USP *in vitro* assay procedure. Protamine sulfate did not neutralize all sodium heparin units to the same degree.

A two-phased study[1] was conducted to establish the significance of these observations: (*a*) to ascertain whether the differences observed in the *in vitro* assay paralleled the *in vivo* response; and (*b*) to compare the ability of protamine sulfate to neutralize heparin samples derived from different source materials, different potencies, or different manufacturing processes. These factors were not always known by previous investigators.

[1] Assays associated with the first-phase study were performed by Eli Lilly and Co. Assays for the second-phase study were performed by Cohelfred Laboratories, Inc., The Upjohn Co., and Eli Lilly and Co.

Table I—*In Vitro* Assay Data[a]

Heparin Source	Potency, USP Heparin Units/mg. Number of Assays	Potency, USP Heparin Units/mg. Mean ± *SE*	Heparin Units Neutralized/mg. Protamine Sulfate Number of Assays	Heparin Units Neutralized/mg. Protamine Sulfate Mean ± *SE*	Relative Neutralization, %
Beef lung	27	135.6 ± 1.8	7	84.6 ± 0.9	100
Swine mucosa	6	159.7 ± 3.9	4	114.6 ± 1.6	135

[a] All standard errors are for $P_{0.05}$.

Table II—*In Vivo* Assay Data[a]

	Protamine Sulfate, mg./kg.	Mean Clotting Time, min.	Number of Rabbits
Beef lung heparin, units/kg.			
100	0.174	22.32	10
100	0.292	16.45	10
100	0.554	11.23	20
Swine mucosal heparin, units/kg.			
100	0.174	20.78	10
100	0.292	12.80	10
100	0.554	6.64	20
Controls	—	4.26	10

[a] A comparison of the two curves, calculated as per Finney (4), established that protamine sulfate neutralized 30% more heparin units derived from swine mucosa than from beef lung.

FIRST-PHASE STUDY

Two samples of sodium heparin, one of lung origin and one of mucosal origin, were assigned potencies by the USP assay procedure. They were then evaluated by the USP *in vitro* protamine sulfate neutralization assay.

Methods—The sodium heparin potencies were assigned by the official procedure (USP XVII, p. 611). The *in vitro* heparin neutralizations were determined by the official procedure (USP XVII, p. 539).

The *in vivo* heparin neutralization method is a modification of a procedure described by Gross (2). Rabbits were injected intravenously with 100 units/kg. of sodium heparin. Two and one-half minutes later, they were injected with sufficient protamine sulfate to produce a dose–response curve as measured by clotting time. Clotting time was determined 7.5 min. after the administration of protamine sulfate by using the capillary tube method described by Peterson and Mills (3).

Results and Discussion—A summary of the *in vitro* assay data is found in Table I; a summary of the *in vivo* assay data is found in Table II. The *in vitro* assays established that 1 mg. of protamine sulfate neutralized 84.6 heparin USP units of lung origin. (This approximates the 85.8 units expected by the USP *in vitro* neutralization assay as specified under *Heparin preparation*.) One milligram of protamine sulfate neutralized 114.6 heparin USP units of mucosal origin (35% more than for lung heparin). The *in vivo* study demonstrated a 30% difference for the two materials. These results indicate that neutralization values, obtained by the USP *in vitro* assay, are indicative of the *in vivo* response in rabbits.

Table III—Data from Second-Phase Protamine Sulfate–Sodium Heparin Neutralization Study

Sodium Heparin Lot No.	Laboratory	Sulfur, %	Potency, USP Heparin Units/mg. "As Is" Number of Assays	Range	Mean	Heparin Units Neutralized/mg. Protamine Sulfate Number of Assays	Range	Mean	Relative Neutralization, %[a]
			Source—Beef, Lung						
C	A	—	3	114–117	116	2	86	86	100
C	B	—	2	116	116	4	84–94	88	103
C	C	12.24	4	115–117	115	2	86	86	100
	Average				116			87	101
B	A	—	2	129–133	131	3	92	92	107
B	B	—	5	131–137	133	6	87–97	93	108
B	C	13.15	4	131–137	134	2	87	87	101
	Average				133			91	106
A	A	—	2	150–152	151	2	101–104	103	120
A	B	—	2	157	157	5	101–107	103	120
A	C	13.70	4	157–163	160	4	86–91	89	104
	Average				156			98	115
			Source—Beef, Mucosa						
11148-27.5	A	—	4	98	98	2	77	77	90
11148-27.5	B	—	3	102–106	105	3	76–79	77	90
11148-27.5	C	11.91	4	101–104	103	5	78–81	79	92
	Average				102[b]			78	91
11148-27	A	—	3	126–128	127	2	95	95	110
11148-27	B	—	3	130–135	133	4	96–102	98	114
11148-27	C	11.42	4	128–130	129	3	91–94	93	108
	Average				130			95	111
			Source—Swine, Mucosa						
LP09138L	A	—	4	128–132	130	2	94	94	110
LP09138L	B	—	3	132–138	136	3	94–104	98	114
LP09138L	C	11.60	4	134–139	136	2	104	104	121
	Average				134			99	115
LP09138	A	—	4	153–155	154	2	109–112	111	129
LP09138	B	—	5	151–158	154	3	110–117	114	133
LP09138	C	11.65	4	156–160	158	2	107–111	109	127
	Average				155			111	130
LP01299	A	—	8	166–174	169	3	121–127	123	143
LP01299	B	—	3	167–175	170	2	128	128	149
LP01299	C	13.68	4	168–173	170	2	114–117	116	135
	Average				170			122	143
			Source—Swine, Mucosa[c]						
11018-26C	A	—	3	143	143	2	104–107	105	122
11018-26C	B	—	3	144–148	146	5	102–105	105	122
11018-26C	C	12.73	4	141–146	144	2	108–112	110	128
	Average				144			107	124
11208-28	A	—	4	167–171	169	1	116	116	135
11208-28	B	—	3	167–171	170	3	112–115	114	133
11208-28	C	12.45	4	169–174	171	2	111	111	129
	Average				170			114	132
X-10018	A	—	3	177	177	2	125–128	127	148
X-10018	B	—	3	167–177	172	6	114–124	120	140
X-10018	C	12.10	4	177–194	184	2	114–117	116	135
	Average				178			121	141

[a] Relative percent neutralization was determined by dividing number of heparin units neutralized by 85.8 units. [b] Sample 11148-27.5 does not meet the USP potency requirement. [c] Inorganic sulfate used in manufacture.

SECOND-PHASE STUDY

Sodium heparin samples known to be from different sources, of different potencies, and from different manufacturing processes were procured. These were first assigned heparin unit potencies by the USP procedure, and they were then neutralized by the USP protamine sulfate assay. The sulfur content of all materials was also determined[2].

Methods—The sodium heparin potencies and the *in vitro* neutralization assays were performed as previously indicated. After cation removal, sulfur determinations were made by the Schöniger method.

Results and Discussion—A summary of data from the second-phase study is found in Tables III and IV. Among the several so-

Table IV—Summary of Results

Heparin Source	Potency, Units/mg.	Heparin Units Neutralized/mg. Protamine Sulfate	Relative Neutralization, %
Beef lung	116–156	87–98	101–115
Beef mucosa	102–130	78–95	91–111
Swine mucosa	134–178	99–122	115–143

[2] The beef lung heparin was supplied by The Upjohn Co.; the heparin of mucosal origin (swine and beef) was supplied by Cohelfred Laboratories, Inc.; and the protamine sulfate was supplied by Eli Lilly and Co.

dium heparin samples examined that met the USP potency requirement, 1 mg. of protamine sulfate neutralized from 87 to 122 sodium heparin USP units, a maximum variation of 42%.

Within each type of sodium heparin examined, an apparent correlation was observed between heparin units neutralized by 1 mg. protamine sulfate and heparin potency. No correlation was observed between sulfur content and the neutralization values.

SUMMARY

Although all types of sodium heparin materials were not included, and no attempt was made to quantitate the parameters examined, namely tissue, species, potency, and process, this study established that there is a significant difference in the neutralization of different types of sodium heparin by protamine sulfate.

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Hexahydrocoenzyme Q_4 in Pseudohypertrophic Muscular Dystrophy

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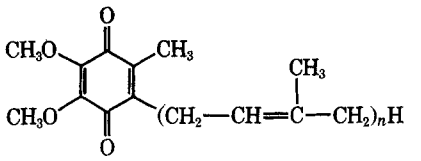
Abstract □ Hexahydrocoenzyme Q_4, 250 mg. per day for 8 months followed by 1000 mg. per day for 4 months, did not improve muscle strength or alter serum and urine creatine and creatinine, serum creatine phosphokinase, or aldolase, or a battery of other clinical and laboratory indexes including oral glucose tolerance and associated insulin, growth hormone, and inorganic phosphorus levels in 19 boys with pseudohypertrophic muscular dystrophy of the Duchenne type. The failure to observe beneficial or other changes during the ingestion of hexahydrocoenzyme Q_4 might have been because of: (*a*) the intrinsic inactivity of the substance in Duchenne dystrophy, and (*b*) the low dose level, particularly if this dystrophy should be one of the vitamin-dependent diseases of genetic nature which involves vitamins of both the water- and oil-soluble category. In any case, the effective dosage of hexahydrocoenzyme Q_4 for the genetic muscular dystrophy of mice in a previously reported study was approximately 10–50 times that used in this clinical study. The dosage for the mice was "massive" in terms of their body content of coenzyme Q. Hence, the studies herein reported do not exclude the possibility that higher homologs of the coenzyme Q group, *i.e.*, Q_5–Q_{10}, might have beneficial effects in human muscular dystrophy. In such trials, coenzyme Q_{10} would certainly be the most important, since it is present in human tissues.

Keyphrases □ Muscular dystrophy, pseudohypertrophic—treatment with hexahydrocoenzyme Q_4, evaluation □ Hexahydrocoenzyme Q_4—evaluation of use in pseudohypertrophic muscular dystrophy □ Coenzyme Q_4 homologs—evaluated in muscular dystrophy treatment

Coenzyme Q_{10}, a relatively new vitamin (1), is widely distributed in mammalian species. Certain rodent tissue such as that of mice and rats may contain mostly coenzyme Q_9 but also some Q_{10}. The normal members of the coenzyme Q group, represented by I, differ in the number, *n*, of the isoprenoid units in the side chain. From the viewpoint of mammalian metabolism, coenzymes Q_9 and Q_{10} may be regarded in the category of the oil-soluble group of vitamins such as vitamin A and vitamin D.



I

n = 1–10

n = 10 for human tissue

n = $-CH_2CH{=}C(CH_3){-}CH_2(CH_2{-}CH_2{-}CH(CH_3){-}CH_2)_3H$ for hexahydrocoenzyme Q_4

Coenzyme Q_{10} is naturally present in the human body. It was found in every organ and tissue analyzed (2) and, presumably, is in every cell of the human body that has mitochondria. Coenzyme Q_{10} is a component of the bioenergetic reactions of respiration and coupled oxidative phosphorylation which reside in the inner mitochondrial membrane. The presence of coenzyme Q_{10} in these electron-transfer processes is indispensable, and the molecule has the general structural specificities of a vitamin. It is evident that increasing deficiencies of coenzyme Q_{10} would be increasingly deleterious to health and be reflected by some nature of disease, depending upon the distribution of the deficiency in the body.

Human muscle tissue and heart tissue obtained at autopsy from three individuals showed 20–30 mcg. CoQ_{10}/gram of wet weight (gww) tissue and 50–80 mcg. CoQ_{10}, respectively (2).