Improved Bioassay for Glucagon by Continuous Glucose Monitoring

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Abstract □ A simplified and rapid *in vivo* bioassay for glucagon is described. The test involves continuous monitoring of blood glucose which makes possible an exact rendering of the glucose peaks induced by intravenous injection of glucagon. Two injections of known amounts of glucagon (0.002 and 0.004 U) are followed by an intravenous injection of a solution containing an unknown amount of glucagon for testing. The whole procedure can be completed in 8 h. The interassay variability of 6 bioassay procedures was ±12.4%. The intra-assay variability was ±3%.

Keyphrases □ Glucagon—*in vivo* bioassay, continuous glucose monitoring, dogs □ Glucose monitoring—glucagon, *in vivo* bioassay, dogs □ *In vivo* bioassay—glucagon, continuous glucose monitoring, dogs

Measurement of glucagon and its synthetic analogues by radioimmunoassay (1–3) does not necessarily reflect their biological activity, since there is no relationship between the binding affinity of glucagons to antibodies and their biological activity. There are several established *in vitro* bioassays for glucagon that are based on: (*a*) the *in vitro* release of glucose by liver slices; (*b*) the stimulation of adenyl cyclase in subcellular particles of the dog liver (4); and (*c*) the relaxing action on strips of rabbit renal artery (5).

Although these *in vitro* bioassays are more specific than the immunoassays, they do not necessarily reflect biological activity *in vivo*. Consequently, the USP bioassay of glucagon requires measurements of the *in vivo* hyperglycemic effect of glucagon (6). The USP bioassay also calls for multiple assays of the glucose levels in eight fasting adult cats and requires four intravenous injections of glucagon to each cat (two injections of a standard solution and two injections of assay samples). Two blood samples are taken after each injection.

The purpose of this study is to describe a simplified and rapid *in vivo* bioassay for glucagon, which can be completed in 8 h and requires only one dog.

EXPERIMENTAL SECTION

Animals—Six female hound dogs, weighing 17 kg, were used. The test was repeated six times on each dog at 2-week intervals. The dogs were anesthetized with intravenous sodium pentobarbital[1] (150-mg initial dose, 25 mg every 30 min for maintenance). Continuous glucose monitoring was then initiated, using a nonthrombogenic catheterization of the front paw.

Instruments—The glucose monitoring instrument consisted of systems for blood withdrawal and glucose measurement. The blood withdrawal system included a disposable sterile intravenous catheter connected to a peristaltic pump. Coating the inside of the catheter with triiododecylmethylammonium chloride complexed with heparin created a nonthrombogenic surface. The blood that was withdrawn continuously (at a rate of 8 mL/h) from a vein through the nonthrombogenic catheter was diluted in the plexiglass mixing chamber with 6 volumes of simultaneously withdrawn phosphate buffer solution (0.15 M, pH 7.4) and moved into the sensory chamber.

[1] Nembutal.

Table I—Six Repeated Bioassays of 0.003 U Glucagon after Intravenous Injection of Glucagon

	Blood Glucose Levels, mg/dL			
Dogs	Peak 1, 0.002 U	Peak 2, 0.004 U	Peak 3, 0.003 U	Bioactivity of 0.003 U of Glucagon
1	129	155	147	0.00 33
2	125	148	133	0.00 26
3	109	121	115	0.00 28
4	95	105	97	0.00 23
5	108	128	120	0.00 30
6	108	135	124	0.00 30
Mean	112.3	132	122.7	0.00 28
±*SD*	12.5	18.2	16.8	0.00 035
CV, %	11.12	13.70	13.60	12.4

The glucose measuring system included a plexiglass mixing chamber, a glucose-sensing probe, a digital display and a microprocessor-controlled digital graphic recorder. The glucose probe was a Clark electrode (7). A membrane impregnated with glucose oxidase covered the tip of the probe. Enzymatic oxidation of glucose diffusing through the glucose oxidase membrane generated hydrogen peroxide at a rate proportional to the concentration of glucose. Thus, current flow through the electrode was linearly related to the concentration of blood glucose. The level of blood glucose was exhibited by a digital display and by the digital graphic recorder.

Reagents—A glucagon standard solution for intravenous injection was prepared in accordance with the USP (6).

Bioassay Method—The hyperglycemic peak induced by intravenous injection of glucagon was accurately depicted in real time, using the nonthrombogenic glucose monitor. The assay consisted of comparing the hyperglycemic peak, induced by an unknown amount of glucagon, to the peaks induced by two intravenous injections of a known amount of glucagon.

The blood level of glucose was first monitored for at least 0.5 h in order to establish a baseline level of glucose. After establishing a steady baseline, the dog was given the first standard amount of glucagon. The glucagon was administered as an intravenous injection in a rapid bolus which was immediately flushed with 10 mL of saline. The first peak of glucose was recorded by the glucose monitor within 14 min after the injection of glucagon. A period of 41 min following a peak was allowed for restabilization of the glucose baseline. This procedure was then repeated using a second standard amount of glucagon. The second standard was usually higher than the first standard. After recording the second peak of glucose, a third injection of an unknown amount of glucagon was administered. The level of "unknown" glucagon was then calculated by linear regression of semilogarithmic paper of the three peak levels P_1, P_2, and P_3.

RESULTS

Interassay Variability—The bioassay was repeated six times using standards of 0.002 and 0.004 U of glucagon and an injection of 0.003 U to represent the unknown. The results are given in Table I. The interassay variability of the six consecutive assays was 12.4%.

Intra-assay Variability—The intra-assay variability of the bioassay was measured by comparing the variability of five consecutive peaks by a constant amount of glucagon. Two assays of intra-assay variabilities were carried out: one with 0.002 U, the other with 0.004 U. The results are recorded in Tables

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Table II—Variability of Peaks after Repeated Intravenous Injection of 0.002 U of Glucagon

	Blood Glucose Levels, mg/dL					Intra-assay Variability		
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Mean	±*SD*	*CV*, %
1	107	105	111	105	104	106.4	2.79	2.62
2	101	102	98	97	93	98.2	3.56	3.62
3	131	130	127	129	127	128.2	1.79	1.38
4	96	111	114	113	110	108.8	7.33	6.73
5	100	104	101	101	102	101.6	1.50	1.47
6	118	111	121	114	112	115.2	4.21	3.65
Mean	108.8	110.5	112	109.8	108	109.8	3.53	3.24
±*SD*	13.2	10.25	10.25	11.4	11.4			
CV, %	12.13	9.27	9.00	10.5	10.5			

Table III—Variability of Peak after Repeated Intravenous Injection of 0.004 U of Glucagon

	Blood Glucose Levels, mg/dL					Intra-assay Variability		
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Mean	±*SD*	*CV*, %
1	125	122	119	119	123	121.6	2.60	2.14
2	123	124	116	115	117	119	4.18	3.51
3	108	102	97	104	107	103.6	4.40	4.24
4	106	95	110	107	109	105.4	6.02	5.71
5	141	145	154	146	145	146.2	4.77	3.26
6	112	116	119	118	118	116.6	2.60	2.24
Mean	119.2	117.3	119.2	118.2	114.8	117.6	4.09	3.52
±*SD*	13.20	17.70	19.08	14.90	13.60			
CV, %	11.07	15.08	16.12	12.60	11.35			

II and III. The intra-assay variability of the assay using 0.002 U of glucagon was 3.2%. The intra-assay variability of the assay using 0.004 U was 3.5%.

The time interval from the intravenous injection of 0.002 U of glucagon to the peak level of glucose was 10.0 ± 1.2 min (mean ±1 *SD*), while the time to peak of 0.004 U glucagon was 11.9 ± 2.5 min. The peak level of glucose was maintained for 100 ± 69 s after 0.002 U of glucagon and 92 ± 49 s after 0.004 U of glucagon.

DISCUSSION

This new bioassay of glucagon is very similar to a previously published improved bioassay for insulin (8). Similar to the insulin bioassay, the improved accuracy of the bioassay for glucagon is based on the ability to obtain a continuous and almost instantaneous record of the changing levels of blood glucose. The continuous glucose monitor made possible an accurate determination of the glucagon-induced peaks of blood glucose, even though it lasted only for a few minutes and occurred at unpredictable times.

The USP bioassay depends on sampling of blood for glucose at predetermined times (6) which almost never occurred at the precise moment of the glucose peak. We have found the actual peaks of blood glucose, which were induced by a repeated but constant dose of glucagon, to be markedly consistent. The time to peak, on the other hand, was highly variable in relation to its duration. Thus, it is obvious that the USP bioassay, which depends on blood sampling for glucose levels at predetermined times, is inherently less accurate. This new bioassay procedure would be more practical, less expensive, and more accurate than the USP bioassay for the standardization of USP preparations of glucagon, provided the actual dose used for injection is adjusted to range from 0.002 to 0.004 U.

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