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Utilization of a Glycol-Stabilized Liquid NAD for the Measurement of Three Enzymes on the GEMSAEC

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Some analytical parameters have been investigated for a recently described stabilized liquid coenzyme technology in which water-free NAD is dissolved in 1,2 propanediol. Correlation for 108 specimens assayed for AST, ALT and LD with a reference method in which glycol-based NAD was absent was ≧ 0.998 with near identical reproducibility over a period of at least 107 days. Mean recovery of exogenous serum enzymes in this linear kinetic assay is 103%. With the option of mixing only the volume of reagent needed for the enzymatic assay, waste can be eliminated as compared to more costly preparations stabilized by lyophilization. Hazards from an impure water supply are avoided since no reconstituting volume is required.

NICOTINAMIDE ADENINE NUCLEOTIDE (NAD) is universally accepted as coenzyme of choice for a number of assays performed in the clinical laboratory. Nevertheless a number of disadvantages militate against its use: it is unstable in some aqueous solutions; it is unstable as a dried product in a moist environment; the extinction coefficient is affected by trace heavy metals normally present in distilled water. Most laboratories purchase this material lyophilized even though freeze-drying contributes to the high cost passed on to the consumer. Recently Modrovitch [1] reported that salted $NADH_2$, when solubilized in a glycol derivative, maintained a one-year shelf life provided that residual water was removed. In this communication we evaluate such a liquid polyol coenzymes preparation available commercially by documenting its performance on three substrate assays normally requested in the emergency section of a busy hospital laboratory.

MATERIALS AND METHODS

Reagents. Stabilized liquid substrates as well as glycol-stabilized NAD are obtained from Medical Analysis Systems, Camarillo, Co., 93010 and stored at refrigerator temperatures. When combined the working reaction concentrations are estimated to be: 248 mM L-alanine, 8.5 mM α-ketoglutarate, 745 U/L LDH and 71 μM NADH for alanine aminotransferase assay (ALT; EC 2.6.1.2); 143 mM L-aspartate, 8.5 mM α-ketoglutarate, 745 U/L MDH and 71 μM NADH for aspartate aminotransferase assay (AST; 2.6.1.1.); 79 mM L-lactic acid, 3899 μM NAD for lactate dehydrogenase assay (LD; EC 1.1.1.27). Correlative reference reagents in which the combined substrate-coenzyme mixture is stabilized by lyophilization are obtained from Smith Kline Instruments, Inc., Sunnyvale, CA., 94086. Reaction concentrations are described by the manufacturer to be: 112 mM L-alanine, 9.0 mM α-ketoglutarate, 333 U/L LDH and 240 μM NADH for ALT assay; 110 mM aspartate, 10 mM α-ketoglutarate, 600 U/L MDH and 200 μM NADH for AST assay; 93 mM lactate and 3000 μM NAD for LD assay.

Instrumentation. A GEMSAEC centrifugal analyzer (Electro-Nucleonics, Inc., Fairfield, N.J. 07008) equipped with Rotoloader IV is used for all measurements.

Procedure. All reactions are performed at 30°C at 340 nm in "Auto Rate" reaction mode. The settings for ALT and AST are identical: 50 μl sample volume, 200 μl flush volume, 400 μl reagent volume using 40% of a 1 ml pump. Initial readings occurred at 90 s, with 60 s reading intervals for 4 consecutive readings. The settings for LD are fixed at the following volumes: 20 μl sample, 80 μl flush (via 40% of 200 μl pump), 600 μl reagent (via 60% of 1 ml pump). Initial readings occurred at 30 s, with a 30 s reading interval for 5 consecutive readings. Reference working substrate was reconstituted from ion-free water, while the liquid stabilized NAD was added to its substrate at about 1:150 (V/V) to insure the availability of excess coenzyme.

RESULTS AND DISCUSSION

It is conceivable that glycol derivatives might interfere with some enzymatic procedures. Therefore several serum specimens were analyzed by the proposed method and the comparative reference method in which glycol-based NADH was absent. Specimens were collected from a normal and abnormal hospital population in equal portions for ALT, AST and LD assay, stored at −20°C and assayed as described. Comparative statistical parameters by regression analysis as illustrated in Table 1 reveal good correlation and no statistically significant differences in the technology for 108 specimen assays over the observed range extending from normal to more than ten-fold the upper limit of normal.

Reproducibility. Within-run precision was evaluated from coefficient of variation data by 5 duplicate

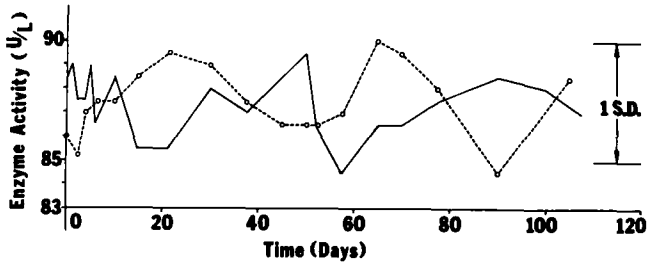

Fig. 1 — Comparative effect of a liquid stabilized (———) and daily reconstituted lyophilized (- - - -) coenzyme preparation on day-to-day quality control of LD.

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TABLE 1

STATISTICAL COMPARATIVE PARAMETERS FOR ENZYME ASSAY WITH (Y) AND WITHOUT (X) A GLYCOL-STABILIZED NAD

	ALT	AST	LD
number of specimens	36	36	35
quantitation range (U/L)	7–139	6–470	49–553
correlation coefficient, r	0.999	0.998	0.999
slope, m, estimating equation	1.02	0.99	0.96
intercept, b, estimating equation	−0.21	−0.42	5.59
T-probability	0.0001	0.0001	0.0001

TABLE 2

RECOVERY OF EXOGENOUS SERUM ENZYMES (U/L) OBTAINED BY LIQUID STABILIZED COENZYME ASSAY.

assay description	A	B	C	D
AST	83	106.8	109.5	103
ALT	53	84.8	88	104
LD	222	267	273	102
			mean	103

Where A = endogenous enzyme present
B = predetermined enzyme present (control serum + A)
C = enzymatic activity by the proposed method
D = recovery in % = C/B X 100

assays on 2 specimens each selected from the clinically normal and abnormal range. For ALT, this data yielded mean values of 6.5% and 0.3% respectively; for AST 3.9% and 0.8% respectively; for LD, 1.0% and 0.5% respectively. Correlative values obtained using pre-lyophilized coenzyme were 4.4% and 0.6% respectively for ALT; 2.0% and 1.4% respectively for AST; 1.9% and 0.4% respectively for LD. In no instance are these values, averaged over the analytical range, greater than similar values obtained from a peer group summary of a recent enzymology survey [2]. Between-assay precision on 2 normal and 2 abnormal specimens on 5 consecutive days yielded the following mean values for glycol stabilized and non-stabilized coenzyme respectively; for ALT 4.3% and 5.2%; for AST 4.1% and 3.4%; for LD 5.7% and 2.4%. The similarity in the pattern of reproducibility is evidence that the liquid-stabilized coenzymes preparation is near to identical in its behaviour with the pre-lyophilized product. Comparative long-run variation over a period of 107 days for LD is illustrated in Figure 1. It is consistently similar for ALT and AST as well.

Recovery. Since reagent concentrations are appreciably different in the reference and proposed mixtures it was necessary to examine the linearity of the kinetic assay using liquid stabilized coenzyme. As reference materials a normal human serum base enriched with enzyme activity derived from porcine and bovine heart (DuPont Elevated Enzyme Verifier, E. I. duPont Co., Wilmington, DE 19898) was mixed with increasing quantities of normal human based serum (DuPont Normal Enzyme Verifier). The proportional dilution effect is close to linear for ALT, AST and LD as illustrated in Figure 2. Enzyme recovery was determined on a pooled serum specimen collected from several hospital patients who were being diagnosed for liver disease. Pre-standardized enriched control serum (DuPont Elevated Enzyme Verifier) was added to each specimen and the recovery was calculated as illustrated in Table 2. Since essentially all exogenous enzyme was accounted for it is presumed that the glycol based coenzyme has no inhibitory effect on the assay.

To date, the majority of commercially available preparations of NAD are lyophilized which inherently leads to a number of problems including lot-to-lot variation in the packaging as well as variation in measurement of the reconstituting fluid and occasional presence of particulate matter due to incom-

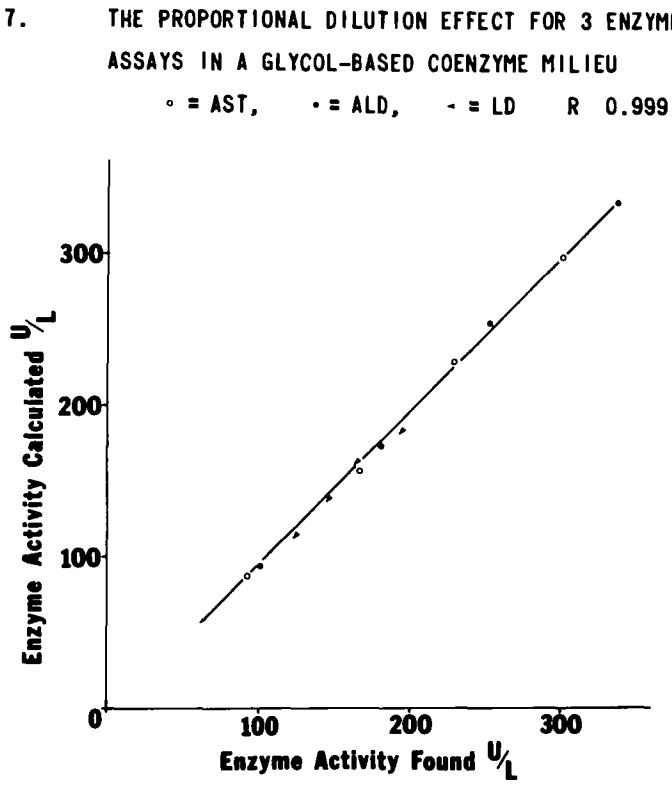

Fig. 2 — The proportional dilution effect for 3 enzyme assays in a glycol-based coenzyme milieu. (○) AST, (●) ALT, (►), LD r = 0.999.

plete dissolution of components. All these difficulties can be avoided by solvating the coenzyme in a protective glycol to produce a stabilized homogeneous liquid essentially water free and stored at refrigerator temperature. Frajola and Maurukas [3] have recently documented the stability of human serum enriched with ethylene glycol for quality control. Apparently the effect is somewhat similar when applied to coenzyme preparations as described in this communication in which it is presumed that 6.65 g/L NADH is dissolved in spectroscopically pure 1,2 propanediol to which 10% V/V molecular sieve is added to remove the water [1]. In our hands analysis of the product available from Medical Analysis Systems revealed only trace water concentration at about 0.13% V/V and glycol concentration of about 99.0% as analyzed by gas chromatography.

With this technology it is to some advantage that workers can avoid waste by proceeding directly to the assay run without having to reconstitute material just prior to use. Thus coenzyme and substrate can be combined *as needed*. Because of recent restrictions on reagent water duly specified [4] this technology should prove especially attractive to smaller laboratories which service a remote area where water purity presents a problem. At this writing trace quantities of calcium, copper, iron, manganese and some other substances have an appreciable effect on inhibiting enzyme activity [5].

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