Eur J Cancer Clin Oncol, Vol. 21, No. 10, pp. 1219–1223, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00

Effect of Sodium Molybdate on Protein Measurements: Quality Control Aspects of Steroid Hormone Receptor Assays

SHANTHI RAAM* and TIMOTHY TEIXEIRA
Tufts University Medical Cancer Unit, Lemuel Shattuck Hospital, Boston, MA, U.S.A.

Abstract—*The quantity of steroid hormone receptors in human mammary carcinomas is of importance for predicting clinical response to hormone modes of therapy. There have been several reports describing an increase in the yield of cytosolic receptors when sodium molybdate is included in the tumor cytosol. Since the quantitative units for receptors are* fmol *hormone bound per* mg *cytosolic protein, we examined whether this oxyanion had an interfering or enhancing effect on two methods of protein measurements: those of Bio-Rad and of Lowry. Our results showed that sodium molybdate, even when present in a* 5 mM *concentration in the protein solution, interferes with the color development by both reagents. This effect of molybdate is consistent, reproducible and statistically significant. We present the data to caution the investigators to be aware of this phenomenon while interpreting their own data on the molybdate effect on steroid receptors.*

INTRODUCTION

OUR LABORATORY has been involved in the study of quality control for steroid hormone receptor assays [1–3] and was the first to organize a pilot quality assurance study for the Eastern Cooperative Oncology Group [1]. Several investigators have reported an increase in the yield of cytosolic steroid hormone receptors [4–8] when the tissues are homogenized in buffers containing sodium molybdate. A paradoxical observation has also been reported by Lazier *et al.* [9]; while inclusion of 20 mM concentration of the oxyanion in the homogenizing buffer resulted in an increase in the cytosolic estrogen receptors, the authors noted a marked decrease both in the KCl-extractable nuclear protein and nuclear estradiol binding sites. Based on these observations, it was concluded that the measurement of estrogen and progesterone binding sites in human breast cancer cytosols could be significantly influenced by the presence or absence of the oxyanion in the buffer used by different laboratories. Several hypotheses have been proposed to explain the mechanism of action of sodium molybdate which leads to a higher yield of hormone binding sites [4–9]. Prior to exploring the mechanism of action we decided to examine whether the oxyanion had any effect on two widely used methods of protein measurements: the Lowry technique [10], in which a copper reagent is utilized, and the Bio-Rad technique (Bio-Rad Laboratories, Richmond, CA, U.S.A.), which involves the use of the dye, Coomassie blue. We observed that the color development with these reagents was significantly reduced in the presence of the oxyanion and our experimental data are presented in this report.

MATERIALS AND METHODS

Protein reference standard solution SRM927 was obtained from the National Bureau of Standards, Washington, DC. Protein standard for constructing the standard calibration curves was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All reagents required for Lowry's procedure of protein estimation, anhydrous sodium carbonate, cupric sulfate, sodium tartarate and Folin-ciocalteu reagent, were

Accepted 18 April 1985.
*To whom all correspondence should be addressed at: Tufts University Medical Cancer Unit, Lemuel Shattuck Hospital, 170 Morton Street, Boston, MA 02130, U.S.A.

purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Bio-Rad dye reagent was obtained from Bio-Rad Laboratories. Sodium molybdate (ACS certified) was purchased from Fisher Scientific.

All protein standard solutions containing 100, 200, 300, 400 or 500 μg of protein/ml were prepared by diluting the protein stock solution obtained from Sigma Chemical Co. For quality assurance, the protein content of this Sigma stock was periodically calibrated using protein standard solutions prepared from the reference standard SRM927 obtained from the National Bureau of Standards. Normal saline (sterile, irrigation saline, 0.9% NaCl) was used for diluting the protein solutions.

Protein measurement using Bio-Rad reagent

Bio-Rad dye concentrate was diluted according to manufacturer's instructions in distilled water, filtered and stored. Fifty microliters each of the protein standard solutions (100–500 μg of protein/ml) were mixed with 2.5 ml of the Bio-Rad reagent. After 20 min incubation at room temperature, optical density measurements (OD) were taken at 595 nm using a Gilford spectrophotometer.

Mico-Lowry procedure

One hundred microliters of the protein standard solutions were mixed with 3 ml of the Lowry reagent (copper sulfate, sodium tartarate and 2% sodium carbonate in 0.1 N sodium hydroxide). After 10 min at room temperature 0.3 ml of 1 N phenol reagent were added to each solution and mixed. After 30 min the optical density measurements at 640 nm were taken.

Preparation of matched pairs of protein standards with or without sodium molybdate

Two 1-ml aliquots were taken from each of the protein standard solutions (100–500 μg/ml). Twenty microliters were removed from each of the 1-ml aliquots. To one set 20 μl of saline were added; to another set 20 μl of 1 M sodium molybdate solution were added to yield a set of standards containing 20 mM final concentration of sodium molybdate. To prepare protein standards containing 5, 10 or 15 mM sodium molybdate, 5, 10 or 15 μl of 1 M solution along with 15, 10 or 5 μl of saline were added respectively to bring the total volume of the added solution to 20 μl. This procedure of introducing molybdate into the same protein solutions was adopted in order to eliminate the variability often encountered when the solutions were diluted individually from the stock. Statistical significance of the data were estimated by *t* test [11]. All experiments were done in replicates of three or four.

Effect of the presence of 20 mM *sodium molybdate in protein solutions on protein measurements*

Two sets of experiments were conducted to examine whether inclusion of 20 mM sodium molybdate in the protein standard solutions affects the protein measurements by either interfering with or enhancing the color development.

Protein standard solutions containing 100, 200, 300, 400 or 500 μg were made with or without 20 mM sodium molybdate as described previously. Optical density measurements were compared in solutions with and without the oxyanion after treatment of the samples following Bio-Rad's procedure (Table 1) or by Lowry's procedure (Table 2) of protein measurements. Each experimental variable was in triplicate.

Comparison of the effect of the presence of 5, 10, 15 *and* 20 mM *sodium molybdate in protein solutions on protein measurements*

Two hundred micrograms per milliliter of protein solution, either with no molybdate or with 5, 10, 15 or 20 mM sodium molybdate, were prepared in triplicate on three different occasions. After the addition of Bio-Rad reagents, the optical density measurements were taken at 595 nm. The values obtained for solutions without the

Table 1. *Protein measurements using Bio-Rad reagent: effect of* 20 mM *sodium molybdate on color development*

Protein concentration (μg/ml)	Optical density measurements at 595 nm* No molybdate (mean ± S.D.)	With molybdate (mean ± S.D.)	*t* value†
100	0.103 ± 0.0087	0.054 ± 0.0079	9.3396
200	0.194 ± 0.0081	0.1006 ± 0.0107	15.5900
300	0.282 ± 0.0198	0.149 ± 0.0216	11.1950
400	0.359 ± 0.0250	0.206 ± 0.0177	11.1920
500	0.451 ± 0.0096	0.279 ± 0.0124	11.7800

*No difference was noted in the optical density measurements of the reagent blanks with and without molybdate.

†Degrees of freedom = 10. All values exceed 4.587; $P > 0.001$.

Table 2. Protein measurements using Lowry's procedure: influence of 20 mM sodium molybdate on color development

Protein concentration (μg/ml)	Optical density measurements at 640 nm* No molybdate (mean ± S.D.)	With molybdate (mean ± S.D.)	*t* value†
100	0.050 ± 0.0038	0.040 ± 0.0025	3.846
200	0.100 ± 0.0045	0.080 ± 0.0026	6.660
300	0.140 ± 0.0048	0.120 ± 0.0076	4.440
400‡	0.190 ± 0.0091	0.160 ± 0.0036	5.319
500‡	0.210 ± 0.0103	0.210 ± 0.0103	-

*Reagent blanks with or without molybdate yielded the same optical density measurements.

†Degrees of freedom = 6. All values exceeded 3.707; $P > 0.01$; none significant at the 0.001 level All *t* values are lower than 5.959.

‡Note plateau effect in molybdate-free solutions.

*Table 3. Effect of different concentrations of sodium molybdate on protein measurements by Bio-Rad technique (protein concentration of the solution = 200 μg/ml)**

	Set 1 OD/595 nm (mean ± S.D.)	Set 2 OD/595 nm (mean ± S.D.)	Set 3 OD/595 nm (mean ± S.D.)
No molybdate	0.260 ± 0.0073	0.252 ± 0.0041	0.341 ± 0.0047
+5 mM molybdate	0.156 ± 0.0049	0.139 ± 0.0043	0.254 ± 0.0033
+10 mM molybdate	0.123 ± 0.0048	0.126 ± 0.0059	0.223 ± 0.0052
+15 mM molybdate	0.125 ± 0.0025	0.144 ± 0.0022	0.196 ± 0.0026
+20 mM molybdate	0.144 ± 0.0039	0.136 ± 0.0040	0.206 ± 0.0046

t values were calculated for matched, pairs in each set: molybdate-free controls vs +5, 10, 15 or 20 mM. $P > 0.001$, with all *t* values exceeding 8.610; d.f. = 4. *t* values were also calculated for each set comparing the OD units obtained for solution with 5 vs 20 mM sodium molybdate. Set 1, $t = 2.706$, N.S.; set 2, $t = 0.7143$, N.S.; and set 3, $t = 12.00$, $P < 0.001$.

Conclusion: The effect of molybdate, namely, a significant decrease in color development, is consistent and reproducible. This effect is not concentration dependent in the range 5-20 mM of sodium molybdate.

*Three different sets of protein solutions with or without molybdate were prepared as described in the text and analyzed separately.

oxyanion were compared with the optical density measurements of the same solutions with different concentrations of the oxyanion. In addition, the optical density values of protein solutions with 5 mM molybdate were compared with those containing 20 mM molybdate. The data analyzed by *t* test are presented in Table 3.

In order to assess the day-to-day variations in the optical density measurements, one set of standards, with or without sodium molybdate (5, 10 or 20 mM), were prepared on day 1 and analyzed on three different occasions. Measurements were done in replicates of five. Statistical significance of the day to-day variability in OD units obtained for each of the solutions was estimated from the *t* values calculated by comparing the highest and lowest mean ± standard deviation values obtained for each of the solutions (Table 4).

RESULTS

Effect of 20 mM *sodium molybdate on protein measurements*

Inclusion of 20 mM sodium molybdate in the protein solutions resulted in a significant reduction in color development with both Lowry and Bio-Rad reagents (Tables 1 and 2). A decrease in the optical density units was observed in molybdate-containing solutions when compared with the OD units obtained for the oxyanion-free solutions. All protein concentrations, 100-500 μg/ml range, showed a similar effect with Bio-Rad procedure (Table 1). With Lowry's technique the molybdate effect was evident in four of the protein standard solutions (100-400 μg/ml). There was no difference in the OD units obtained for molybdate-free and molybdate-containing 500 μg/ml protein solution. However, it is also important to note the plateau effect seen between OD units of

the molybdate-free, 400 and 500 μg/ml solutions (Table 2) with the Lowry reagents.

Effect of different concentrations of sodium molybdate

Table 3 contains the OD values obtained with Bio-Rad dye for 200 μg/ml protein solutions either without or with 5, 10, 15 or 20 mM concentration of sodium molybdate. Inclusion of molybdate in the protein standards even at a 5 mM concentration effectively reduces the intensity of color development. It is also important to note that when measuring proteins using Bio-Rad's procedure, 50 μl of the protein standards are further diluted with 2.5 ml of the dye solution, resulting in a 51-fold dilution of molybdate in the final mixture. In Lowry's procedure the dilution is 34-fold since 100 μl of the protein solutions are diluted with a total volume of 3.3 ml of the Lowry reagent dye solutions.

The intra-assay and the inter-assay variabilities were quite minimal as seen from the OD values and the results of the *t* test (Tables 3 and 4); therefore, molybdate-associated reduction in color development is quite significant, and this effect is reproducible. Three separate experiments yielded the same results (Table 3); the same set of protein solutions analyzed on three different occasions also yielded reproducible results (Table 4).

DISCUSSION

Our results have clearly established that sodium molybdate, even at 5 mM concentration in the 50 μl aliquots of protein solution taken for quantifying protein, interferes with the color development, resulting in a significanct decrease in the optical density values obtained with both the Bio-Rad and Lowry reagents. This decrease in optical density was not proportionate to the concentration of the oxyanion, nor was the effect of 5 mM sodium molybdate significantly different from that of 20 mM molybdate (Table 3). An apparent lack of such an effect only for 500 μg/ml protein solution measured by Lowry's method (Table 2) is evidently due to an insufficient amount of Lowry reagent in the system for measuring 500 μg/ml protein.

Investigators who are studying the effect of molybdate on steroid hormone receptors and those who are performing routine quantitative analysis of cytosolic steroid hormone receptors in clinical samples should be aware of the interference of molybdate ion on the protein measurements. The quantitative units for steroid hormone receptors are fmol of ligand binding sites per mg cytosolic protein. A laboratory using molybdate in the buffer would obtain a lower protein value for a cytosol due to the presence of molybdate and, therefore, a higher quantity of steroid binding sites per mg cytosolic protein. Thus, for the same tumor cytosol two different values for hormone binding could be obtained, causing a problem for quality assurance. We found that diluting the cytosol samples containing 10 or 20 mM concentration of sodium molybdate at least 13-fold with molybdate-free buffer or saline which reduced the concentration of molybdate in the 50 μl aliquots taken for protein measurement nullified the interference of molybdate with color development. A better procedure would be to quantify the protein based on a standard curve constructed using protein solutions containing the oxyanion at the same concentration as cytosols.

The authors wish to emphasize that by presenting the data discussed in this report they *do not imply* that the sodium molybdate has no effect on the yield of cytosolic steroid hormone receptors. They simply wish to caution other

Table 4. Significance of inter-assay variability of molybdate effect (protein concentration of the solution = 300 μg/ml)*

	Optical density units (mean ± S.D.)			
	Day 1	Day 2	Day 3	*t* value†
No molybdate	0.363 ± 0.0190	0.339 ± 0.019	0.319 ± 0.011	1.987
+5 mM molybdate	0.238 ± 0.0344	0.285 ± 0.033	0.255 ± 0.025	1.978
+10 mM molybdate	0.202 ± 0.0219	0.232 ± 0.039	0.211 ± 0.013	1.340
+20 mM molybdate	0.197 ± 0.019	0.235 ± 0.0299	0.196 ± 0.029	1.866

The decrease in OD units seen between the molybdate-free and molybdate-containing solutions were all statistically significant for all levels of molybdate on all three days. *t* values of all the matched pairs, OD units of molybdate-free control, vs OD units of either 5, 10 or 20 mM molybdate-containing solutions exceeded 3.355, $P > 0.01$.

Conclusion: The molybdate effect is reproducible; there is very little inter-assay variability.

*Protein solutions with or without molybdate were prepared on day 1 and analyzed on three different occasions, each time in replicates of five.

†*t* values were calculated for matched pairs of highest and lowest mean ± S.D. Degrees of freedom = 8. None of the *t* values are significant at the 0.05 level.

investigators to take this effect of molybdate on protein measurements into consideration while interpreting their own data. It will also be worthwhile to examine the effect of molybdate ion on the DNA quantification method of Burton [12], which is commonly used by several investigators involved in steroid hormone research for measuring nuclear receptors.

Acknowledgements—The authors wish to acknowledge the technical help of Mark L. Harnois.

REFERENCES

1. Raam S, Gelman R, Cohen JL *et al.* Estrogen receptor assay: interlaboratory and intralaboratory variations in the measurement of receptors using dextran-coated charcoal technique: a study sponsored by ECOG. *Eur J Cancer* 1981, **17**, 643-649.
2. Raam S, Gelman R, Faulkner J, White GM, Cohen JL. Quality control for estrogen receptor quantification by dextran-coated charcoal assay: a single laboratory's experience. *Breast Cancer Res Treat* 1982, **2**, 111-117.
3. Cohen JL, Raam S, Gelman R, Wittliff JC. A blinded study of inter- and intra-laboratory variations in the performance of estrogen receptor assay. In: Sarfaty GA, Nash AR, Keightley DD, eds. *Estrogen Receptor Assays in Breast Cancer: Laboratory Discrepancies and Quality Assurance*. New York, Masson Publishing U.S.A., Inc., 1981, 43-56.
4. Gaubert GM, Blancucci S, Shyamala G. A comparison of the cytoplasmic estrogen receptors of mammary gland from virgin and lactating mice. *Endrocinology* 1982, **110**, 683-685.
5. Krozowski ZS, Murphy LC. Stabilization of the cytoplasmic estrogen receptors by molybdate. *J Steroid Biochem* 1981, **14**, 363-366.
6. Anderson KM, Phelan J, Marogel M, Hendrikson C, Economou S. Sodium molybdate increases the amount of progesterone and estrogen receptors detected in certain human breast cancer cytosols. *Steroids* 1980, **35**, 273-280.
7. Gaubert CM, Tremblay RR, Dube JY. Effect of sodium molybdate on cytosolic androgen receptors in rat prostate. *J Steroid Biochem* 1980, **13**, 931-936.
8. Hawkins EF, Leiskovsky G, Markland FS Jr. Molybdate and the measurement of androgen receptors in prostate cancer. *J Clin Endocrinol Metab* 1981, **53**, 456-458.
9. Lazier CB, Richman J, Lonegran K. The effect of molybdate on the intracellular distribution of estrogen receptors in mammary tumors. *Breast Cancer Res Treat* 1984, **4**, 19-26.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the Folin phenol reagent. *J Biol Chem* 1951, **193**, 265-275.
11. Clark TC, Schkade LL. *Statistical Analysis for Administrative Decisions* (2nd edn). Cincinnati, OH, South-Western Publishing Co., 1974.
12. Burton K. A study of the condition and mechanism of the diphenylamine reaction for the colormetric estimation of deoxyribonucleic acid. *Biochem J* 1956, **62**, 315-323.