

QUANTITATIVE DETERMINATION OF HORMONE
METABOLITES AND GLYCOGEN BY USING
LIQUID SCINTILLATION QUENCHING METHOD

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ABSTRACT

The colour quenching in liquid scintillation counting has been a major problem and requires an internal or external quenching correction for each coloured sample. However, colour quenching presents a good and a rapid method for the quantitation of those compounds for which appropriate colour could be developed. But this technique has not been applied for the estimation of hormone metabolites. The objective of present work was to use the colour quenching technique and develop it into a method for the estimation of hormone metabolites.

Sealed glass ampules containing ^3H -labelled source in toluene base scintillation fluid was placed, precisely centred, in a scintillation vial containing about 5.0 ml of solution of the developed colour to be quantitated. The vial was counted in a liquid scintillation counter (Packard Model 3380) to find out the extent of colour quenching.

The colour produced by the Kober colour reaction for estriol (1) and the phenol - sulphuric acid reaction for glycogen (2) gave linear decrease in the counting rate of the scintillation source. The critical evaluation of the method and its application in quantitative determination of urinary estriol and tissue glycogen, in this study, demonstrate that the sensitiveness, accuracy and precision of the scintillation quenching quantitation equals that of the spectrophotometric method.

INTRODUCTION

In liquid scintillation counting, the colour quenching is usually linear. The quenching results in the decrease in counting rate which is proportional to colour. The linearity in quenching may be exhibited by any colour which absorbs in the region of the spectrum in which the maximum response is shown by the phototubes used in the liquid scintillation spectrometer. Making use of this linearity, it has been possible to utilize the liquid scintillation quenching for the quantitation of lipid mass (3).

The use of liquid scintillation quenching technique for the quantitation of colour presents a good and a rapid method for the estimation of those compounds for which appropriate colour could be developed. However, this technique has not been applied for the estimation of hormone metabolites. The objective of the present work was to use the colour quenching technique and develop it into a method for the estimation of hormone metabolites. This rapid technique has been developed to standardise methods for the estimation of urinary estriol during pregnancy and glycogen in rat uterus, liver and muscle.

MATERIALS AND METHODS

Scintillation Source. About 28,000 cpm of ^3H -labelled source in 0.5 ml of simple scintillation fluid (toluene base) was pipetted in a small pyrex glass ampule. The glass ampule was sealed. Care was taken to prevent the scintillation medium from being warmed up during sealing. A large number of such glass ampules containing the β -emitting source were prepared. Each ampule was placed in a glass counting vial (20 ml) containing 5.0 ml of toluene. The position of the ampule within the vial was precisely centred with the help of a polyethylene adaptor and counted in a liquid scintillation counter (Packard Model 3380). Ampules containing 27,900 to 28,100 cpm were selected. For the purpose of colour quantitation, the developed colour product (about 5.0 ml) was taken in the counting vial, an ampule containing scintillation

source was placed and counted as above .

Quantitation of various colour reactions.

Kober colour reaction for Estriol. Taking various levels of estriol (0 to 40 μg), Kober colour reaction was performed according to Brown's method (1). The optical density of the developed pink colour was taken at 500, 542 and 576 nm (Zeiss PMQ II Spectrophotometer). The corrected optical densities [$2 \times \text{O.D. at } 542 - (\text{O.D. at } 500 + 576)$] were plotted against the various amounts of estriol (μg). The duplicate samples were transferred to counting vials. The glass ampules containing known scintillation source (28,000 cpm of ^3H) were placed centrally in each vial and counted in the liquid scintillation counter. The rate of colour quenching (cpm of ^3H obtained) for increasing amount of estriol was plotted on a graph paper.

Phenol-Sulphuric acid reaction for Glycogen. Colour reaction for various amounts of standard glycogen (0 to 80 μg) was performed according to Dubois et al (2). The glycogen was taken in 2.0 ml of distilled water, 0.1 ml of 80% phenol solution in distilled water added and immediately followed by the addition of 5.0 ml of conc. Sulphuric acid. It was mixed, allowed to stand in ice water for 30 minutes and optical density taken at 490 nm. The duplicate samples were subjected to scintillation quenching. The optical densities and cpm of ^3H obtained were plotted against the various concentrations of glycogen.

Hydrolysis, extraction and purification of urinary estriol. An aliquot (2.0 ml) of 24-hour male urine or pregnancy urine was taken in Kober tube, conc. hydrochloric acid was added until the pH was 2 or below (pH paper) and 2.5 g of sodium chloride was added. Ethyl acetate (4.0 ml) was added and vortexed for two minutes at room temperature. After centrifugation, 2.0 ml of the solvent phase, representing one ml of urine, was taken, dried under nitrogen and dissolved in ether.

Urine extract contains substances which yield

colour products on chemical reaction. These disturbing chromogens are effectively removed by the method of Brown (1). This involved the washing of ether extract with sodium carbonate solution (pH 10.5), sodium hydroxide solution, sodium bicarbonate solution and finally with distilled water. The ether extract was then partitioned between benzene-light petrolèum and water. The water extract containing estriol was acidified and the estriol re-extracted with ether. The ether extract was finally purified on alumina columns.

Extraction of tissue glycogen. The method of Dubois et al (2) was adopted for the extraction of tissue glycogen. To every 100 to 200 mg of tissue (rat uterus, liver and muscle) 1.0 ml of 30% potassium hydroxide was added, and heated in boiling water for 10 minutes. After cooling, 0.5 ml of 2% sodium sulphate and 1.2 ml of 95% alcohol was added and kept for overnight at room temperature. After centrifugation, the supernatant was discarded and the residue was dissolved in known amount of distilled water.

RESULTS.

Evaluation of the assay method.

Sensitivity Test. The linearity of the relationship between estriol and the corrected optical density and the counting rate is presented in Fig. 1. Fig. 2 shows the relationship between various concentrations of glycogen and optical density and the counting rate. The counting rate of the ^3H -radioactive scintillation source decreased by the colour intensity. The sensitivity of colour quenching curve for both estriol and glycogen was good and quite comparable with their respective absorbance. The range could be further extended to at least 60 μg for estriol and 160 μg for glycogen by scintillation colour quenching. Slopes were also identical on repeated observations.

Accuracy Test. The accuracy of a quantitative method is usually studied by means of "recovery experiments" in which determinations are made on the material being analysed before and after the addition of known amounts of the substance under investigation. Since both the urinary

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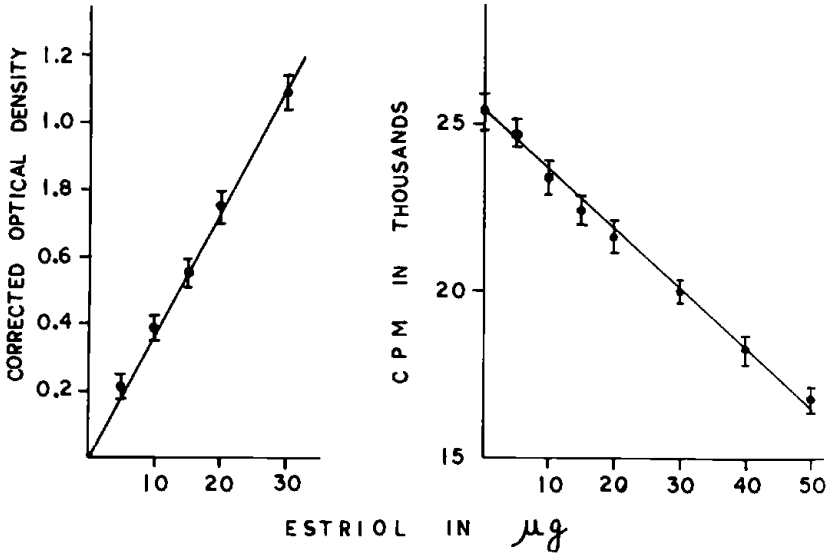


Fig. 1 Estriol Standard Curve : The dose response curve by the spectrophotometric method ($542\text{ m}\mu$) and the scintillation quenching method.

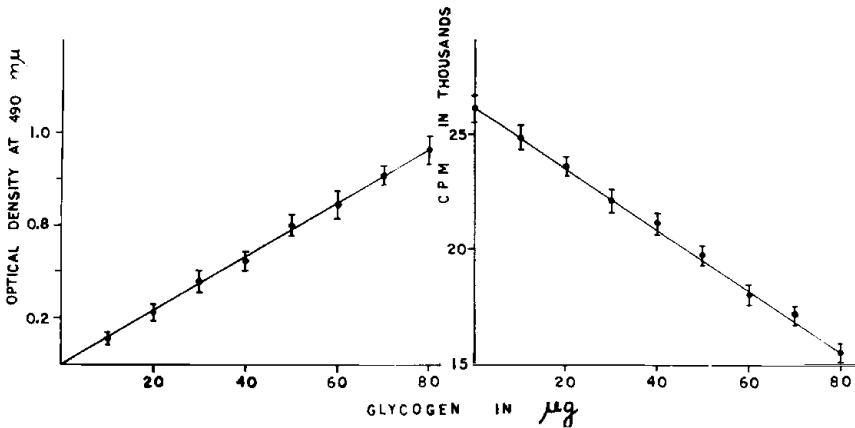


Fig. 2 Glycogen Standard Curve : The dose response curve by the spectrophotometric method ($490\text{ m}\mu$) and the scintillation quenching method.

estriol and tissue glycogen estimation by optical density method are well established and being extensively used by many laboratories, no attempt has been made to analyse the recovery during urine extraction, purification etc. or tissue glycogen preparation. Our major aim was to study the accuracy of quantitation of developed colour by scintillation quenching and to look into its possible replacement for spectrophotometric method.

To each 1.0 ml of male urine extract (urine hydrolysed, extracted and purified as described above) 5 to 25 μg of estriol was added. After Kober colour reaction, the developed colour was quantitated by scintillation quenching method. Since the value of estriol in 1 ml male urine (about 7.0 μg in 24-hour urine) was below the practical detection limit, no subtraction for endogenous estriol was made. After addition of varying amounts of estriol to the male urine extract, the amount of estriol was measured by scintillation quenching method. It is presented in Fig.3. It may be observed that the scintillation quenching and the concentration of the estriol added had a linear relationship. The method was quite accurate and reproducible for the quantitation of estriol.

Similarly, 10 to 50 μg of glycogen was added to aliquots from a tissue glycogen preparation. The colour was developed and quantitated by scintillation quenching. The amount of glycogen already present in each aliquot ($11.8 \pm 1.01 \mu\text{g}$) was subtracted from each observation. Fig. 4 shows the amount of glycogen measured for every addition of standard glycogen before colour reaction. Accuracy of the scintillation quenching method in quantitation of glycogen was also demonstrable.

Precision Test. An estimate of precision of a chemical assay method is usually obtained by carrying out multiple determinations on the same sample. Precision is usually expressed as the standard deviation of replicate determinations.

Multiple determinations of the urinary estriol in 4 samples from varying weeks of pregnancy by both spectrophotometric and scintillation quenching methods have been presented in Table I. The standard deviations

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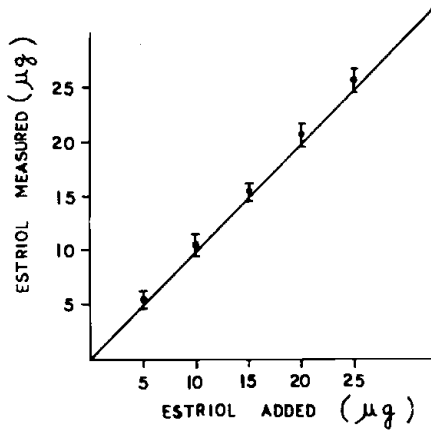


Fig. 3 Accuracy of the urinary estriol quantitation by scintillation quenching method.

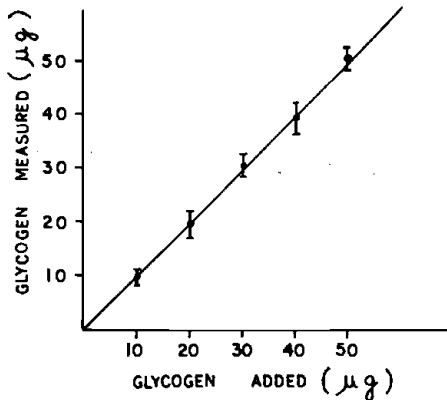


Fig. 4 Accuracy of the tissue glycogen quantitation by scintillation quenching method.

TABLE- I

The Precision of Urinary Estriol quantitation by classical optical density method and scintillation quenching method

Pregnant women	Optical Density Method			Scintillation Quenching Method		
	MEAN (μg)	S.D.	Coefficient of variation (%)	MEAN (μg)	S.D.	Coefficient of variation (%)
S.S.	4.7	0.49	10.4	5.3	0.59	11.1
S.D.	7.1	0.69	9.7	7.2	0.90	12.5
G.A.	15.4	0.91	5.9	16.9	1.02	6.0
K.R.	20.0	0.94	4.7	20.6	1.05	5.1

and the coefficient of variations (%) for the two methods may be observed to be quite comparable. The coefficient of variation for the new method ranged between 5.1 to 12.5 per cent. This may indicate a high degree of precision.

Table II shows the multiple estimations of glycogen in various tissue glycogen preparations by both methods. It may be observed that the precision of the glycogen quantitation by scintillation quenching method was also comparable to the spectrophotometric method.

Urinary estriol estimation in pregnant women. Large number of 24-hour urine from women at various weeks of pregnancy were analysed for estriol. The values obtained by scintillation quenching method has been compared with those obtained by spectrophotometric method (Fig. 5). It may be observed that estriol ($\mu\text{g/ml}$ of urine) quantitated by scintillation quenching method was not different from that obtained by spectrophotometric determination.

Tissue glycogen estimation. Tissue glycogen was prepared from rat uterus, liver and muscle. Also, to evaluate the influence of different concentrations of glycogen, varying aliquots from these tissue preparations were quantitated for glycogen by both methods. Ranging between 10 to 80 μg of glycogen were quantitated by scintillation quenching method and the values did not differ from that of the spectrophotometric method (Fig. 6).

DISCUSSION

The scintillation quenching method for the quantitation of colour has been evaluated and applied for the estimation of urinary estriol and tissue glycogen. The method is as sensitive as that of the spectrophotometric procedure. Measurement of varying concentrations of estriol and glycogen demonstrated that the quantitation by scintillation quenching method is quite accurate. Repeated estimations on a number of samples indicated that the precision of the quenching method equals that of the spectrophotometric method.

TABLE 11

The Precision of Tissue Glycogen quantitation by classical optical density method and scintillation quenching method

Tissue preparations	Optical Density Method			Scintillation Quenching Method		
	MEAN (µg)	S.D.	Coefficient of variation (%)	MEAN (µg)	S.D.	Coefficient of variation (%)
1. Rat liver	10.6	1.26	11.9	10.3	1.32	12.8
2. Rat muscle	12.5	0.85	6.8	11.8	1.30	11.0
3. Rat muscle	18.8	1.03	5.5	19.1	1.33	7.0
4. Rat muscle	21.7	1.54	7.1	21.1	1.44	6.8
5. Rat liver	34.6	3.38	9.8	34.1	3.62	10.6

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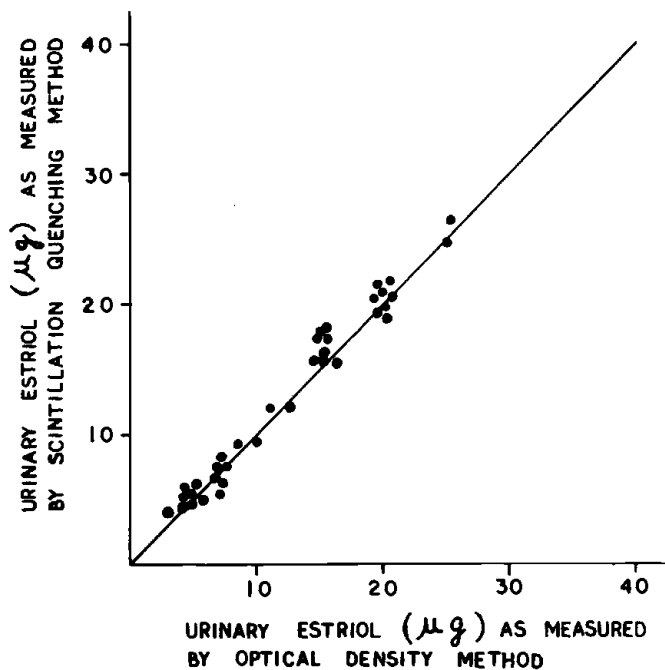


Fig. 5 Quantitation of Urinary Estriol: The accuracy of scintillation quenching method as compared with that of the classical spectrophotometric method.

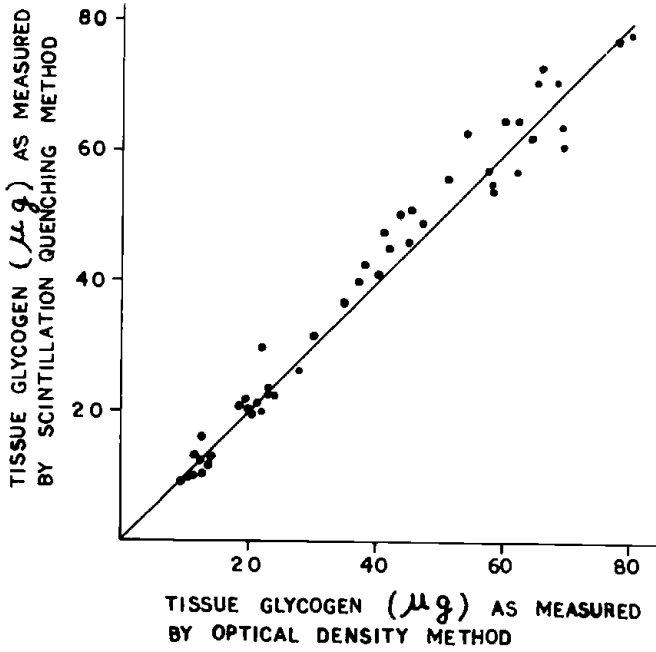


Fig. 6 Quantitation of Tissue Glycogen : The accuracy of scintillation quenching method as compared with that of the classical spectrophotometric method,

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The evaluation of scintillation quenching for the quantitation of coloured products and its application in estimating the urinary estriol in pregnant women and the tissue glycogen may suggest that the scintillation quenching method is a good alternative to that of the spectrophotometric procedure.

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