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A Clinical Method for Determination of Riboflavin Standard in Urine after a Test Dose and the Riboflavin Excretion of Clinically Normal Subjects.

By

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In an article "Om B₂-vitaminernas relation till stomatitis angularis (cheilosis, perlèche)" (Regarding the relationship between B₂ vitamins and angular cheilosis, see bibliography) the author has previously made an account of the principal investigations within this department. These investigations are, however, purely clinical and the diagnosis has here been made exclusively by means of clinical symptoms and on examination of the reaction to the treatment of riboflavin. This kind of diagnostics is the only one used at present, as previously there have been no clinically available methods for the determination of a standard. EMMERIE's and SNELL & STRONG's methods most often used at experimental investigations are too complicated for clinical use.

Investigations in the determination of riboflavin in urine have previously been executed *e. g.* by EMMERIE, AXELROD, NAJJAR & HOLT etc. with varying results.

In order to be able to make a closer study of the etiological relationship between angular cheilosis, glossitis and other diseases of the oral tissues on one hand and of riboflavin deficiency on the other, the author has been anxious to find a clinically useful method for the standard determination of riboflavin in man.

In 1944 and 1947, A. GOTH has given accounts of a simple method worked out by him, which the author has found to be worth a closer examination. The basis of the method is on the principle of gravity and on the determination per cent of the riboflavin in urine.

A Brief Description of Goth's Method.

The determination is carried out after an overnight fast. The person to be examined makes a urine specimen, whereupon 5 mg riboflavin are injected intramuscularly. Two hours later, still fasting, another urine specimen is taken. The entire volume of this last excreted urine is now measured and noted. 1 ml of each urine is diluted with 9 ml distilled water in two test tubes. Under the filtrated ultraviolet light, the first urine is titrated with a test solution, containing 20 γ riboflavin per ml, until its fluorescence equals that of the second specimen of urine. The percentage of riboflavin excreted is estimated.

GOTH believes that if more than 30 % of the injected riboflavin is excreted there will be *no deficiency* but below 20 % there is a *decided deficiency*.

As the method, however, has apparently not been thoroughly examined and the account made by GOTH of his material does not give a true account of its reliability, the author has found it necessary to begin with an examination in defence of the method. When the result of this has been statistically worked out, the author has passed on to the study of riboflavin excretion in the urine of subjects without clinical symptoms of riboflavin deficiency, and here the excretion has been determined according to the present method.

Below there is a brief account of these investigations. For further details see "Svensk Tandläkare-Tidskrift" 1950:5:1.

The Author's Investigations.

(By normal subjects are meant persons without clinical symptoms of riboflavin deficiency.)

As service of light an Original Hanau, ultra-violet ray-lamp for analyses, has been used. For test solutions and injections Amp. lactoflavin Astra, Sweden, has been used.

The investigation consists of two parts:

1. Examination of the method.
2. Examinations of the excretion of normal subjects
 - a) on their normal diet
 - b) after parenteral injection of riboflavin.

Examination of the Method.

Possibility of retitration.

The first series of investigations intended exclusively to give an idea of the possibility of retitration and was executed in the following way:

From a specimen of urine 1 ml was taken into each of two test tubes. Dilution by distilled water 1:10. In one of the specimens an assistant put a quantity (unknown to me) of test solution (containing 20 γ per ml), after which I titrated the other specimen in the manner described above, until I obtained an identical fluorescence.

Result (10 determinations):

To judge from these determinations the possibility of retitration seems to be good. No determination shows a larger error than 9 %, 8 determinations are below 5 %.

Influence of the milieu of urine.

The author proceeded to an examination of GOTH's statement that during the two hours passing between the first and the second urine specimen, the milieu of urine does not undergo such a change that the analysis will be disturbed by it. On this occasion a series of determinations similar to that mentioned above was made but the author now used two urine specimens from the same person taken at two hours' interval after an overnight fast. For this series the urine of two persons to be tested was used.

The result of this series (13 determinations) shows that the error has not increased when I titrated 2 urine specimens taken at two hours' interval. No determination shows a larger error than 7.5 % and in 10 determinations the error is below 5 %. Consequently the result is somewhat better than in the preceding series, probably owing to a greater dexterity at the determination of colour.

The result must be considered as positive. The urine has obviously not undergone such a change as to influence the result of the determination.

The error of the method.

In order to obtain a material as representative as possible for the determination of the size of the error of method, another series of determinations was finally made, arranged in the following way:

The author made 21 double tests with a riboflavin standard known only by him. For these 42 determinations urine from 4 persons to be tested was used. Each one of these persons gave 2 specimens of urine at two hours' interval after an overnight fast. My assistant, an experienced laboratory nurse, who after this executed the titrations, consequently, did not know what quantities the specimens contained, nor did she know that it was double tests. They were *not* made *successively* but were introduced here and there in the series. Thus all kinds of errors will be represented in this material. After this the result which appears from table 1 was worked out statistically.

Table 1.
(42 tests.)

Test No.	Doses of riboflavin ml.	The titrated quantity of riboflavin	
		I	II
1	18 γ	18.4	17.8
2	40	39.8	44.0
3	36	35.6	35.0
4	26	26.0	26.0
5	14	14.4	14.0
6	32	32.6	32.0
7	16	16.0	16.0
8	52	52.0	50.8
9	8	8.0	8.4
10	18	18.2	18.0
11	44	43.0	43.0
12	34	32.0	32.6
13	32	30.4	30.8
14	14	14.6	14.0
15	44	44.0	48.0
16	26	27.2	26.2
17	30	29.0	31.0
18	19	19.6	19.0
19	28	29.2	26.0
20	54	54.0	52.0
21	60	59.0	60.0

Estimation of the Error of the Method.

(According to table 1.)

The material consists of 21 double tests of injections of riboflavin. The error at the determination refers to:

- 1) accidental errors during the process of titration.
- 2) error in the estimation of the fluorescence.
- 3) variations in the concentration of salt, P_H values etc.

The error in its turn can be divided into:

- 1) systematical error. Whether the double tests differ systematically from the doses.
- 2) accidental error.

Notation: dose $R \gamma$

test I = $r_1 \gamma$

test II = $r_2 \gamma$

$R - r_1$ or $R - r_2 = z_1$

$\frac{100 z_1}{R} = y_1$

Systematical error.

In the material no systematical error can be proved; if there is such an error it is of little importance.

Accidental error.

Provided that: 1) there is no systematical error and 2) the error in R is of little importance, we estimate the error of method in the following way: We make the variable y_1 and estimate its spreading (S_{y_1}); as for good reason we can suppose that y_1 is normally distributed, the error of the method for a single determination equals $S_{y_1} = 3.5$.

The error of method for $\frac{r_1 + r_2}{2}$ (the average value of a double determination) is defined as $\frac{1}{\sqrt{2}} \times S_{y_1} = 2.5$.

Thus the limit of error is:

at single tests $\pm 7 \%$ ($P = 0.95$)

at double tests $\pm 5 \%$ ($P = 0.95$)

of the value found which must be regarded as satisfactory for clinical determinations.

Determinations on Normal Subjects.

The investigation of method having shown that after addition according to this method the riboflavin standard in urine can be decided within $\pm 5 \%$ of the values found, the author passed on to a series of test doses on normal subjects. The motive of this part of the investigation was to get an idea of a) the percentage excretion of these persons on their normal diet (ordinary home food) and b) the percentage excretion after parenteral injection of riboflavin

till "saturation". 112 specimens have been executed on in all 34 subjects to be examined, 17 females and 17 males, ages 20—40 years, mainly students and nurses at the State Dental School in Stockholm. At all the test doses the percentage excretion was estimated on the average value of a double determination and the examination was repeated after 4—6 days. During the whole period of investigation all the persons to be tested took a normal diet of ordinary home food. The specimens were taken after an overnight fast (meat and drink of any kind were not allowed after 6 and 7 o'clock in the evening before the examination). The results of these determinations are collected in the tables 2—5 given below.

Discussion.

Thus of these 34 persons tested without clinically discernible symptoms of riboflavin deficiency no less than 29 persons excreted less than 30 % and 14 persons less than 20 %. According to the standards stated by GOTH a definite deficiency would consequently be present in almost half of the persons to be tested, a remarkably high figure for clinically normal subjects, but by no means unreasonable.

Hypothetically there is, on the other hand, also the possibility that the percentage excretion may normally be below the value of saturation without a deficiency of vitamins *i. e.* that a lower standard would be normal for some persons.

As the investigation hitherto has been limited to principally the staff and the students of the Dental School, the material tested is not representative enough and it is too small for general conclusions of a statistical kind.

On comparing the excretion in per cent between the female and male material tested there is no statistically positive difference.

On studying the differences of the percentage excretion of two injections on the same person (4—6 days' interval) you will find that the so called individual variation is relatively great, probably owing to variations of the riboflavin standard in the daily food. However, it can be seen that the persons who excreted more than 30 % showed a relatively small individual variation. The material is, however, still too small to make definite statistical conclusions about the size of the individual variation.

Table 2.
(17 female persons to be tested in the age of 19—32 years.)

Persons to be tested No.	The riboflavin standard injected	Excreted riboflavin in per cent after two hours	
		Gravity I	Gravity II (4—6 days later)
1	5 mg i. m.	10.3	9.4
2	»	16.0	21.8
3	»	16.3	14.6
4	»	12.1	13.3
5	»	17.5	15.7
6	»	21.4	27.4
7	»	26.8	24.5
8	»	28.2	29.3
9	»	21.0	19.8
10	»	21.2	21.6
11	»	18.9	20.4
12	»	16.5	18.5
13	»	31.6	30.9
14	»	31.4	33.1
15	»	23.7	24.7
16	»	16.1	15.6
17	»	23.9	24.8

To be added the error of method: $\pm 5\%$ of the values found.

Table 3.
(17 male persons to be tested in the age of 21—40 years.)

Persons to be tested No.	The riboflavin standard injected	Excreted riboflavin in per cent after two hours	
		Gravity I	Gravity II (4—6 days later)
18	5 mg i. m.	13.1	18.3
19	»	22.3	26.4
20	»	18.4	17.5
21	»	26.6	24.5
22	»	24.4	28.4
23	»	29.2	27.4
24	»	36.0	35.6
25	»	36.0	34.4
26	»	13.5	14.0
27	»	16.0	11.1
28	»	10.7	13.6
29	»	18.0	21.7
30	»	22.3	25.8
31	»	28.1	25.7
32	»	14.0	18.8
33	»	36.9	35.4
34	»	23.7	26.8

To be added the error of method: $\pm 5\%$ of the values found.

For the purpose of studying the effect of riboflavin supply and trying to obtain an increase of the percentage excretion the investigation was continued with parenteral injection of riboflavin on some persons to be tested.

Examination of the Saturation.

17 persons to be tested, 8 females and 9 males, who during the two other examinations had shown a low percentage excretion, were placed on riboflavin therapeutically. 10 mg riboflavin (Astra) were injected intramuscularly daily for 5—10 days, and the conditions of excretion were studied in doing so. Standard determination was carried out 24 hours at the earliest after such an injection. All the persons to be tested were still on their normal diet.

After this the riboflavin injections were discontinued. The persons to be tested were left to a diet that was estimated to cover their daily need of riboflavin, among other things milk, cheese, eggs, meat. After about one week another specimen was made in order to control whether the standard obtained had been preserved. If, on the whole, this controlling examination showed "status quo", one will be right in considering that there was saturation. The result of these determinations appears from the following tables Nos. 4 and 5.

Table 4.

(8 female persons to be tested.)
no. 1, 2, 3, 4, 5, 9, 10, 11.

Persons to be tested no.	Result of previous test dose		Result after inj. of riboflavin i. m. 10 mg for 5—14 days				Controlling specimen after about 1 week
	I	II	5	7	10	14 days	
1	10.3 %	9.4 %	20.8%	21.9%	24.6%		24 % (about 1 month)
2	16.0	21.8	45.2				42.1
3	16.3	14.6	40.1				36.2
4	12.1	13.3	43.3				41.0
5	17.5	15.7	28.7	29.2			23.5
9	21.0	19.8	27.3	30.0			29.6
10	21.2	21.6	33.1				38.4
11	18.9	20.4	23.4		31.8		33.0

To be added the error of method: $\pm 5\%$ of the values found.

Table 5.
 (9 male persons to be tested.)
 no. 18, 20, 22, 26, 27, 28, 29, 30, 32.

Persons to be tested no.	Result of previous test dose		Result after inj. of riboflavin i. m. 10 mg for 5—14 days				Controlling specimen after about 1 week
	I	II	5	7	10	14 days	
18	13.1 %	18.3 %	32.5%				30.2 %
20	18.4	17.5	45.7				44.0
22	24.4	28.4	39.7				43.8
26	13.5	14.0	27.0		30.4		34.1
27	16.0	11.1	18.3	30.9		28.4	
28	10.7	13.6	25.6	27.4	28.2		30.2
29	18.0	21.7	24.3		31.1		33.7
30	22.3	25.8	36.1				34.9
32	14.0	18.8	25.8	29.9			29.1

To be added the error of method: $\pm 5\%$ of the values found.

Discussion.

On daily injection of riboflavin the percentage excretion rapidly increased, in the beginning, in order to keep almost constant when there seemed to be saturation. The excretion varied from 28—46 % in the material to be tested. One person, number 1, proved to be very resistant and in spite of riboflavin supply, for 14 days, the excretion did not exceed 24.6 %. At executed examination of the blood Hb as well as serum-iron were found to be below the normal values.

A great many of the treated persons to be tested reported spontaneously that they felt an evident improvement of their general well-being. They had earlier felt tired and had had poor appetite, which they had considered as normal because of the time of the year (January—March). The symptoms of fatigue disappeared and the appetite obviously increased. They felt “spry and vital”. The author does not want to infer anything from these subjective statements but they do confirm the suspicions that there has really been a hypovitaminosis and that therefore clinically normal subjects and healthy persons are not a priori the same.

The controlling specimen after about one week showed the standard unchanged except in one case, the person to be tested no. 5.

Conclusion and Summary.

The present study constitutes partly a review of the qualifications of GOTH's method, and partly examinations of the excretion in subjects without clinical symptoms of riboflavin deficiency.

1. The examination of the method shows that with this method it is possible to determine the riboflavin content in urine after a test dose within the limits of error to $\pm 5\%$ ($P = 0.95$) of the found values (double determinations) which must be regarded as completely satisfactory for a clinical method.

2. The study of the excretion in clinically normal subjects shows that about 42 % of the material to be tested, staying on normal diet, excreted less than 20 %.

3. After parenteral injection of riboflavin for 5—10 days the excretion rapidly increased until, judging from the results, there was saturation. The excretion then came up to 28—46 %.

4. Controlling test dose about a week after discontinuing of the injections showed the standard practically unchanged except in one case.

The material tested is not representative enough and is too small that one may make statistically reliable conclusions.

As a summary one can, however, say that the hitherto obtained results prove that the method is worth being examined on a larger clinical material. To get a figure as sure as possible the author recommends double determinations, carried out at a few days' interval and on an unchanged diet.

The author is at present occupied with a continued clinical examination series of material *with clinical symptoms of riboflavin deficiency*, where the riboflavin standard is determined according to the present method.

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Zusammenfassung.

Vorliegende Arbeit ist eine experimentale Untersuchung einer klinischen Methode, Riboflavinmangel beim Menschen zu bestimmen. Die Methode wurde zuerst von A. GOTH eingeführt. Die Bestimmung nimmt nur 2—3 Minuten und wird, nachdem eine Belastungsdosis von 5 mg Riboflavin intramuskulär injiziert worden ist, vorgenommen. Nach Fasten über Nacht liefert die Versuchsperson eine Urinprobe ab, und 5 mg Riboflavin werden intramuskulär injiziert. Zwei Stunden später, immer noch fastend, wird eine neue Urinprobe genommen. Die ganze jetzt ausgesonderte Menge wird gemessen und notiert. 1 ml jeder Urinprobe wird mit 9 ml destilliertem Wasser in zwei Reagenzgläsern verdünnt. Bei filtriertem ultraviolettem Licht wird der erste Urin mit einer Versuchslösung, die 20 γ Riboflavin pro ml enthält, titriert, bis seine Fluoreszenz derjenigen der anderen Urinprobe gleicht. Der Prozentsatz abgesonderten Riboflavines wird berechnet. GOTH ist der Ansicht, dass, wenn über 30 % des injizierten Riboflavines ausgesondert werden, kein Mangel vorliegt, dass aber unter 20 % ein sicherer Mangel vorhanden ist.

Untersuchungen des Verfassers.

Die Untersuchungen des Verfassers zeigen:

1. Es ist mit dieser Methode möglich, den Riboflavingehalt im Urin, nach einer Belastungsdosis, mit einem Fehlermarginal von ± 5 % ($P = 0.95$) zu bestimmen.

2. 34 Personen ohne klinische Symptome eines Riboflavinmangels, die ihre normale Diät einhielten, wurden untersucht. Davon sonderten 29 weniger als 30 % aus, 14 weniger als 20 %.

3. 17 Personen mit niedrigen Exkretionen wurden während 5—10 Tagen mit Riboflavin behandelt (täglich 10 mg Riboflavin intramuskulär). Die Exkretionen stiegen rasch bis zur »Sättigung« wonach sie beinahe konstant blieben. Dann wurden 28—46 % ausgesondert.

4. Die Injektionen wurden eingestellt. Nach einer Woche wurden Kontrollbestimmungen vorgenommen. Der Riboflavingehalt war mit Ausnahme von einem Falle unverändert.

Klinische Versuche mit dieser Methode werden fortgesetzt, um Folgendes zu studieren:

1. Den klinischen Wert der Methode.
2. Das Verhältnis zwischen Riboflavinmangel und Cheilosis angularis, Glossitis und anderen Krankheiten der Mundgewebe

Résumé.

La présente recherche est une investigation expérimentale d'une méthode clinique pour la détermination de manque de riboflavine chez l'homme. D'abord la méthode a été introduite par A. GOTH. La détermination ne prend que 2 à 3 minutes et elle est effectuée après une dose d'épreuve de 5 mg de riboflavine par une injection intra-musculaire. Après jeûne d'une nuit l'homme d'expérience fait un échantillon d'urines et 5 mg de riboflavine sont injectés intra-musculaire. Deux heures plus tard, le sujet toujours jeûnant, encore un échantillon d'urines est pris. Tout le volume excrété est mesuré et noté. 1 ml de chaque échantillon d'urines est dilué par 9 ml d'eau distillé dans deux tubes. Au-dessous de lumière ultra-violette filtrée le premier échantillon d'urines est titré par une solution d'épreuve, contenant 20 γ de riboflavine par ml, jusqu'à ce que la fluorescence soit d'accord avec celle du second échantillon d'urines. Le pourcentage de riboflavine excrétée est estimé. GOTH croit que si plus de 30 % de la riboflavine injecté est excrétée, il n'y a pas de manque, mais moins de 20 % il y a un manque décidé.

Les investigations de l'auteur.

Les investigations de l'auteur montrent ce que suit.

1. Par cette méthode il est possible de déterminer la teneur de riboflavine en urines après une dose d'épreuve dans la limite d'erreur de $\pm 5\%$ ($P = 0.95$).
2. 34 sujets sans symptômes cliniques d'un manque de riboflavine, suivant leur régime normal, ont été éprouvés. De ces sujets 29 ont excrété moins de 30 % dont 14 moins de 20 %.
3. 17 sujet avec une excrétion minime ont été traités par riboflavine pendant 5 à 10 jours (10 mg de riboflavine, intramusculaire

journallement). Les excrétiens ont augmenté rapidement jusqu' à «saturation», après quoi les excrétiens ont été presque constantes. Ensuite 28 à 46 % ont été excrétés.

4. Les injections ont été suspendues. Après une semaine, des déterminations de contrôle ont été effectuées. L'état de la riboflavine restait le même à l'exception d'un cas.

Des investigations cliniques selon cette méthode sont continuées pour l'étude

- 1) de la valeur clinique de cette méthode,
- 2) de la relation entre manque de riboflavine et perlèche, glossite et d'autres lésions des tissus buccaux.

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