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The Determination of Urinary Pregnandiol by Thin-Layer Chromatography, with Quantitative Densitometry: Comparative Study with a Spectrophotometric Method

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E. MOLINA-ANDREU, S. MASDEU, R. M. RAS and J. A. AZNAR. *The Determination of Urinary Pregnandiol by Thin-Layer Chromatography, with Quantitative Densitometry: Comparative Study with a Spectrophotometric Method.* R. esp. Fisiol., 28, 129-134. 1972.

A method for the quantitative determination of urinary pregnandiol is presented, being based on the application of photodensitometry to thin-layer chromatoplates, revealing the steroid spots by means of an unspecific reagent.

A comparison is established with the spectrophotometric method of KLOPPER *et al.*, obtaining a close correlation in values higher than 1.5 mg/1000 ml. The reasons for the deficient correlation obtained in concentrations lower than the above mentioned one ase discussed in this work.

The possible advantages it can offer in its application to routinary laboratories are also pointed out.

Among the methods suggested for the determination of the urinary Pregnandiol (Pregnane-3-α, 20-α-diol), the most sensitive and precise are those using gas-liquid chromatography. However, they take a long time and are not suitable for a simultaneous handling of a large number of samples. This makes them little appropriate for use in routine laboratories, which must attend a great number of requests.

The spectrophotometric methods, with purification of the extract by column chromatography, especially that of KLOPPER *et al.* (7), have been widely used for years, although they also demand a great length of time. In our experience, up to 5 days are necessary in order to process simultaneously a set of samples which, in the hands of a single technician, can hardly include more than 14-16 ones.

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In this respect, we consider most interesting for their application to the routinary biochemical laboratory (especially for the vigilance of ovarian cycles), those methods based on the separation of the urinary Pregnandiol by thin-layer chromatography, with direct evaluation of the spots obtained by means of an adequate detector.

Among its advantages, we can point out the following: speed, saving up material, simplicity of operation and possibility of simultaneously processing a larger number of samples than by using other methods.

With a view to verifying the efficacy of the chromatographic procedure, improving its quantitative reading by the application of photodensitometry to the chromatoplates, we began the present study where a comparison is established with a method of such a widely proved clinical usefullness as that of KLOPPER *et al.*

Materials and Methods

THIN-LAYER CHROMATOGRAPHY

WALDI'S technique (15, 16) is followed, with some slight modifications.

Hydrolysis of pregnandiol glucoronide is obtained with chlorhydric acid (10 ml), acting on 50 ml of the 24 h diuresis, at 100° C.

Extraction is carried out with ethylic ethanol-ether (20:5).

The extract is washed twice with NaOH 1 N, and after that with distilled water up to neutral *p*H.

The dry extract is redissolved in 1 ml of chloroform, from which 25 μl are applied on Silicagel G precoated commercial plates (Merck, Darmstadt).

Standards are parallely applied, containing 1, 3, 5, 8, 10 and 15 μg of pure substance (Pregnandiol, Merck, Darmstadt), from a cloroform solution containing 1 mg per ml.

A run of 15 cm is carried out in chamber saturated with the mixture of chloroform-acetone (90:10).

Revealing of the spots is obtained with phosphomolybdic acid in ethanolic solution at 5 %, spraying in aerosol, with later heating of the plate at 70-80° C.

Densitometric reading was done by transmission, without any previous operation to obtain the translucence of the plate, using the Vitatron TLD 100 equipment.

The organic solvents used had no previous redistillation or purification.

Calculations were carried out on the areas of the peaks obtained in the graphic register, using the usual recipes.

SPECTROPHOTOMETRIC METHOD

We have basically used the method of KLOPPER *et al.* (6, 7) above mentioned, with the following modifications:

The acid hydrolysis of the original procedure was substituted by an enzymatic hydrolysis at *p*H 4.7 (adjusted with sodium acetate-acetic acid buffer, 1 M), taking as the source of the enzyme the digestive juice of *Helix pomatia* (Industrie Biologique Francaise).

The colour reaction was performed for two hours at 37° C, sodium sulphite being added to the sulphuric acid.

Readings were taken at 400, 435 and 470 nm, applying later on Allen's correction formula.

All organic solvents used were submitted to a previous redistillation.

The reproductibility of both methods was studied separately, by means of the duplicate method.

For the study of correlation between both procedures, we used 37 samples of urine, with values from 0.1 to 24.0 mg/1000 ml, each of them divided into two portions which were submitted to the Pregnandiol measure separately with each of the methodical.

Results

Comparative study of the accuracy of both methods

We carry out a separate study of samples containing higher and lower concentrations, respectively, at 1.5 mg/1000 ml, considering that different authors have confirmed that the accuracy of the method of Klopper *et al.* (6, 7) is most deficient in the lower limits of excretion of pregnandiol (3).

It is noticed (Table 1) how in lower concentrations at 1.5 mg/1000 ml, the accuracy of the spectrophotometric method is not good. Comparatively, the chromatographic method shows most satisfactory results.

The spectrophotometric method offers in higher concentrations a more acceptable degree of reproductibility, though slightly lower than the values referred to by others (12). In these limits of concentrations the accuracy of the thin layer can be considered as most similar.

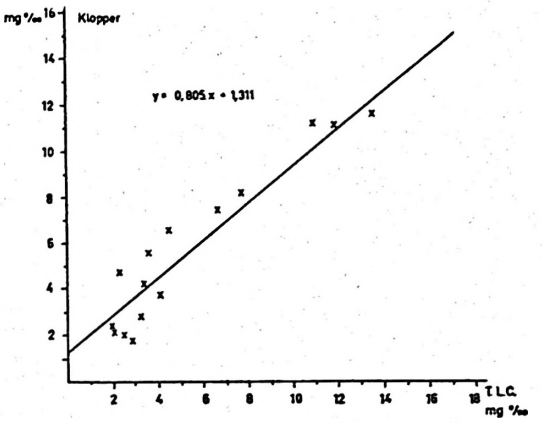

Fig. 1. *Correlation between the compared methods in higher values than 1.5 mg/1000 ml.*

Correlation obtained between both methods

As in the previous paragraph, a separation is established between samples of higher and lower concentrations, at 1.5 mg/1000 ml.

As mentioned above, the same sample of urine was processed by both methods.

The correlation obtained between the

Table I. *Precision of the compared methods.*

	Higher values than 1.5 mg/1000 ml		Lower values than 1.5 mg/1000 ml	
Method	Klopper	TLC	Klopper	TLC
Number of pairs	5	5	7	5
Extreme values	1.9-15.9	3.0-12.0	0.1-1.3	0.3-1.2
Mean value	6.6	7.4	0.4	0.6
Standard deviation	1.16	0.80	0.24	0.07
Coefficient of variation	±17.5 %	±10.8 %	±60.0 %	±12.0 %

Table II. *Correlation between both methods.*

	Higher values than 1.5 mg/1000 ml		Lower values than 1.5 mg/1000 ml	
Number of samples	16	16	21	21
Method	Klopper	TLC	Klopper	TLC
Extreme values	1.8-22.4	1.6-24.0	0.0-1.3	0.1-1.5
Mean value	7.40	7.20	0.70	0.73
r (p=95 %)	0.97 (0.91-0.99)		0.60 (0.22-0.82)	

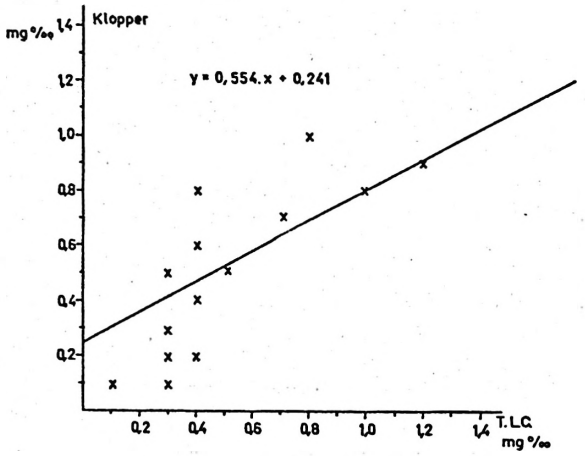

Fig. 2. *Correlation between the compared methods in lower values than 1.5 mg/1000 ml.*

two series of values (Table II) in the limits of higher concentrations is most satisfactory ($r = 0.97$; conficence interval: 0.91-0.99; $p < 0.05$).

On the contrary, in the lower values the correlation obtained is not very good ($r = 0.60$; confidence interval: 0.22-0.82; $p < 0.05$). Likeable reasons for this discrepancy are discussed later on.

The corresponding regression lines, adjusted by the procedure of the minimum squares, are graphically represented (figs. 1 and 2).

Discussion

The spectrophotometric method of KLOPPER *et al.* (6, 7) has undoubtedly been — at least in Europe — the most widely used, in spite of the long time required for its completion.

The specification of this method depends exclusively on an adequate purification of the urine extract, considering that the reaction with sulphuric acid can by no means be considered as specific in the presence of strange substances. The latter, with the original method of KLOPPER *et al.*, can easily reach values of 30-40 % of the optical density read (9, 13).

There are also data showing the discrepancy between the spectrum of absorption in infra-red obtained with pure pregnandiol and with the purified urinary extract in accordance with the method we are dealing with (4).

It seems conclusive that in spite of the great effort required, the purification obtained with this method is not completely effective.

As regards its accuracy, it is most deficient in those concentrations lower than 1.5 mg of pregnandiol per litre of urine.

In spite of the imperfections pointed above, the method of KLOPPER *et al.* is most valuable in its application to the study of the ovarian cycle, clinically speaking, considering that the average values obtained in both phases, follicular and luteal, are significatively different (3).

On top of all this, when in a woman the cycle is ovular, the increase of pregnandiol excretion in the luteal phase is always highly noticeable (10).

In WALDI's original chromatographic method (15, 16), the comparison was visually carried out between the problem spots and those of the standards. This brought about a great proportion of error, since visual appreciation — always most subjective — is influenced by two factors, such as the intensity of colour and the superficial extension of the stain to be evaluated.

In order to reach a better quantification, it has been suggested the scrapping of the silicagel layer and a later application of the reaction with sulphuric acid to the product obtained (12). This improves the results, although they cannot be considered as satisfactory, since besides introducing a further technical difficulty in the practical completion of the method, it reduces its sensitiveness on account of the introduction of a large amount of unspecific chromogens from the chromatoplate into the colorimetric reaction.

In view of the results obtained, we consider as far more interesting the direct densitometry of the stains revealed on the

chromatoplate, due to its greater speed of completion. On the other hand, there is information showing the good reproductibility and excellent recuperations obtained by the application of photodensitometry to chromatoplates (2, 8, 14).

ABRAHAM *et al.* (1) use this method for determining urinary Pregnandiol, by previously revealing the plate with C13Sb, a reactive suggested by PINCUS (11) for the identification of steroids. In accordance with the opinion of the authors of this method, the application of this reactive would make the photodensitometric determination more exact. Nevertheless, the results we have obtained by using phosphomolibdic acid have been good. This is undoubtely due to the good separation obtained with the thin-layer chromatography, which eliminates a maximum of impurities.

Althoug we have been unable to carry out specific studies, we refer to the existing data in previous papers about the degree of purity obtained in pregnandiol separated by thin layer chromatography (1, 12). The data supplied by these authors show a good relation between the pure patterns and the samples of pregnandiol eluted from the plate.

There is still the doubt, at least as far as we are concerned, about up to which point the densitometry in very low excretions of pregnandiol can be considered as specific, bearing in mind that both by using antimony trichloride and phosphomolibdic acid for revealing the plate, you can always find signs of non specific substances which superpose to the steroid spots and which undoubtely will become relatively important when the density of the specific stain is small. It may therefore be interesting an addition of substances that would destroy the unspecific chromogens during the acid hydrolysis (5) although more information would be necessary in order to determine the possible destructive effect they might have on the steroid molecules there existing.

In spite of the above mentioned remarks, the thin-layer densitometric method has enabled us to detect pregnandiol concentrations as low as 0.1-0.3 mg/1000 ml with — this is really important for its clinical application — a degree of accuracy much higher than that supplied by Klopper's method (Table I).

The defficient degree of correlation obtained between both methods in concentrations lower than 1.5 mg/1000 ml, must be logically attributed to the scarce precision shown by the spectrophotometric method in these limits, since the respective average values are quite similar, while the small difference noticed between them is not statistically significative.

As regards the handling of the method by using the thin-layer chromatography, a single well-trained technician is capable of completing 12 tests (3 chromatoplates) in a normal one day's work, in the same series of determinations. This means a speed of operation not surpassed by other methods (12).

If on top of all this we add the excellent qualities of precision and accuracy shown by the method we are dealing with, it apears before us as an altogether ideal technique, for those laboratories where a large number of samples must be processed, thus sparing the more accurate but also more time-consuming ones, e.g. gas-liquid chromatography, for selected cases.

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