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A Simple Accurate Method for Extraction of Estrogenic Substances from Human Urine for Bioassay.*

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In the last few years there have been published a number of methods for the extraction of estrogenic substances from human urine. The methods of Laqueur, Koch, and Kurzrok[1, 2, 3] have been found most satisfactory by this laboratory. Unfortunately the method of Cohen and Marrian[4] has not as yet been made applicable to the determination of estrin in the urine of the non-pregnant female. There is one main disadvantage in the first 3 named methods, and that is the length of time required for the entire extraction process. It would evidently be to great advantage to devise a method which would be simple in procedure, rapid and as satisfactory as the methods of Laqueur, Koch, or Kurzrok. Therefore we present the results of our experimental work.

It is known from the researches of Cohen and Marrian, Zondek[4, 5, 6] and others that a certain portion of the estrogenic substances present in the urine exists in a combined form. In this form it is either biologically inert or non-extractable. This is overcome to a great extent by acid hydrolysis of the specimen. The urine is acidified with 50 cc concentrated hydrochloric acid (35-37%) per liter of urine and then boiled for a period of one hour. Among other workers there has been a considerable difference of opinion as to the amount of acid to use and the length of time the specimen should be heated in the hydrolytic process. Smith and Smith[7] claim to have obtained their maximum increase in potency after boiling 2 hours with 15 vol. % HCl and 4% Zn; whereas Browne, *et al.*,[8] claim that if more than 10 cc concentrated HCl is used per liter of urine, there is de-

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1 Borchardt, Dingemanse, and Laqueur, *Naturwissenschaft*, 1934, **22**, 190.

2 Gallagher, Koch, and Dorfman, PROC. SOC. EXP. BIOL. AND MED., 1936, **33**, 440.

3 Kurzrok and Ratner, *Am. J. Obst. and Gyn.*, 1932, **23**, 689.

4 Cohen and Marrian, *Biochem. J.*, 1934, **28**, 1603.

5 Zondek, B., *Nature*, 1934, **133**, 209.

6 Zondek, B., *Ark. Kemi., Min. O. Geol.*, 1934, part 3, paper 24.

7 Smith, G. V., and Smith, O. W., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 460.

8 Venning, Evelyn, Harkness, and Browne, *J. Biol. Chem.*, 1937, **120**, 225.

struction of estrin. Our experimental work has indicated that the actual method of extraction is a factor whose importance has been insufficiently stressed in the past. Using our method of extraction the results did not show an increase in potency by boiling the urine more than one hour. Some specimens were boiled for periods as long as 4 hours with no definite increase in yield as compared to a control specimen which was boiled for only one hour. However, it was found that boiling the urine for a period of less than one hour did show a definite decrease in potency when tested biologically. If less than 50 cc concentrated HCl was used a longer period of boiling was necessary in order to obtain the same yield as the control specimen which had been boiled with 50 cc of acid. The addition of more than 50 cc HCl per liter did not increase the yield to any noticeable extent.

In order to extract the estrin after hydrolysis, the urine is cooled and shaken vigorously on a mechanical shaker with an equal volume of ethyl ether (U.S.P.) for a period of one hour. This volume of ether and the shaking time were decided upon after numerous extractions had been made with different volumes of ether and different shaking times. For this extraction a "Camp" shaker was used which gives up to 240 circular shakes per minute. It can also be seen from Table I that reëxtraction with ether of the hydrolized urine by shaking one hour yielded no further estrogen.

The emulsion of ether and urine is now put into a separatory funnel and to this mixture is added 30-50 cc sodium taurocholate† (10% sol.) which serves to break up the emulsion. The aqueous layer is discarded and to the ether fraction is added 6 cc sesame oil. The ether is evaporated on a hot water bath. This leaves an oily extract which is ready for bioassay.

In order to determine the accuracy of this extraction process, the

TABLE I.

Extract No.	Process	Yield/Liter R.U.	Yield/Liter I.U.
1	Boil 1 hr, shake 1 hr	40	200
1a	Reextraction of No. 1	0	0
2	Boil 1 hr, shake 1 hr	42.8	214
2a	Reextraction of No. 2	0	0
3	Boil 1 hr, shake 1 hr	37.5	187+
3a	Reextraction of No. 3	0	0
4	Shake 1 hr only	8+	40+

The extractions were carried out on pooled specimen No. 1.

† This substance has been biologically assayed, and does not show any estrogenic activity.

extraction methods of Laqueur, Koch, and Kurzrok were used as comparisons as they are the methods most used in endocrine laboratories.

From 14 to 16 castrated mature female rats were used to assay each original extract. The assays of the material obtained after re-extraction (authors' method) were done on 3 castrated mature female rats. All these animals were primed with crystalline ketohydroxyestrin and are known to respond to a minimum quantity of .5 gamma (5 I.U.).

The dose of extract for each animal was divided into 3 equal injections and given at 9 A.M. and 5 P.M. of the first day and at 9 A.M. of the second day. Vaginal smears were taken 48, 56, and 72 hours after the first injection and stained with thionin and phenol.‡ We considered one Rat Unit as the minimum quantity of extract necessary to produce a positive smear which contains only cornified epithelial cells.

It is noticed from Table II that the method described gives a greater yield than do the methods of Koch or Kurzrok. It will also be noted that Laqueur's method gives a yield approximately the same as the authors' but the time of extraction for bioassay is considerably longer.

Summary. A simple method is described whereby estrogenic substances can be extracted from human urine for bioassay. This method consists of adding 50 cc concentrated HCl (35-37%) per liter of urine; boiling for one hour and then cooling; vigorously shaking on a mechanical shaker for one hour with an equal volume of ethyl ether (U.S.P.); breaking up emulsion with 30-50 cc of sodium taurocholate (10% sol.) and discarding aqueous fraction; adding 6 cc sesame oil to the etherial extract and then evaporating ether on a hot water bath. This method is comparatively accurate and can be accomplished in about 3½ hours, thus making it an excellent routine method in an endocrine laboratory to expedite clinical and experimental work.

TABLE II.

Method	Hours Required for Entire Extraction	Yield/Liter	
		R.U.	I.U.
Laqueur	12	37.5	187
Koch	6	18	90
Kurzrok	53+	13+	66+
Authors'	3+	40	200

The methods were carried out on pooled specimen No. 1.

‡ To 100 cc of a 1% thionin solution was added 1 cc of phenol c.p. This solution was filtered before using.