reagents normally used in the conversion to thiochrome.

2. In solutions of pure aneurin (or in cereal

extracts) the method is accurate to $\pm 20\%$ at concentrations in the region of $0.001 \mu\text{g.}$, the accuracy increasing to $\pm 3\%$ at $0.05 \mu\text{g.}$

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Application of the Millon Reaction to the Determination of Chlorophenols in Body Fluids and Tissues

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The versatile antibacterial usefulness of halogenated phenols makes valuable a specific micro-method for their determination in body fluids and tissues [Zondek, 1942]. The present paper describes a semi-micro-method for *p*-chloro-*m*-xylenol (referred to as CX) and *p*-chloro-*m*-cresol (referred to as CC) in the presence of phenol, e.g. in urine. Patients given CX may excrete this substance in concentrations up to 50 mg./100 ml. The halogenated phenol is allowed to react with Millon reagent at room temperature, the coloured substance so formed being extracted with ether and thus obtained in crystal-clear solution suitable for photometric measurement. Phenol itself under these conditions gives a coloured substance which is only sparingly soluble in ether. The method is therefore highly specific.

Several investigators have used the Millon reaction for the determination of tyrosine, e.g. Folin & Ciocalteu [1927]. The present successful extension of such a method to at least two selected chlorophenols suggests that further investigation would render possible a more general application of the Millon reaction to analytical methods, in which the conventional procedures for the estimation of phenols are often inadequate.

EXPERIMENTAL

Reagents. Reagent 1: A mixture of 50 ml. of a 20% solution of mercuric acetate and 30 ml. of 25% (v/v) HNO_3 ($d. = 1.42$).

Reagent 2: A 1:4 diluted solution of 100 g. of mercury in 200 g. of HNO_3 (Millon reagent).

Standard solutions: 1 g. of CX or CC, twice recrystallized from ethanol and dried *in vacuo*, is

dissolved in 5 ml. N NaOH and diluted with H_2O to 1 l. In amber bottles the solutions are constant for at least 1 month.

A. Procedure with aqueous solutions

Determination of CX. To 15 ml. of test solution in a test-tube of c. 30 ml. capacity are added 2 ml. of reagent 1 and then, if no precipitate has formed, 1 ml. of reagent 2. If a precipitate forms, filter and carry out the test on a measured portion. The mixture is kept at room temperature for 30 min., cooled in ice-water, and 5 ml. of chilled ether added. The colour is brought into the ether phase by shaking, and the test-tube immediately stoppered. Within the next hour the ether extract is transferred to a 0.5 cm. cuvette covered with a glass slide. The extinction value (E) is determined in a Pulfrich photometer with filter S 43. The concentration of the unknown is read by reference to a standard curve prepared by plotting E for known solutions similarly treated. This curve should be renewed for each fresh batch of reagent and should be checked every 2 weeks.

Determination of CC. CC is determined by a somewhat modified procedure. 1 ml. of reagent 1 and 1 ml. of reagent 2 are added to 10 ml. of the test solution. The mixture is incubated for 2 hr. at 37° , cooled in ice-water for 5 min., and shaken with 5 ml. of cold ether. Photometry is carried out within 1 hr. (filter S 50 and 0.5 cm. cuvette).

Results. The colour formed with CX is dirty red in the water phase, but clear yellow in the ethereal solution. With CC the colour is red in water and in ether. Typical standard curves are given in Fig. 1. Within a wide range the relation between E and the concentration of the halogenated phenol is nearly linear. Concentrations of CX or CC greater than 0.5 mg./100 ml. were determined with an error of 5%. Lower concentrations may be determined

by concentrating *CX* or *CC* by extraction from acid solution with a one-third volume of toluene and transferring to a suitable volume of 0.1N NaOH, which is then neutralized with HNO₃. The recovery is practically quantitative. The presence of 0.1N NaNO₃ does not affect the test.

Fig. 1. Dependence of the amount of colour developed with Millon reagent on the quantity of chlorophenol employed. *CC*=*p*-chloro-*m*-cresol; *CX*=*p*-chloro-*m*-xlenol.

Discussion of procedure. By transference from the water phase into ether the coloured substance is concentrated, and at the same time clear solutions suitable for colorimetry are obtained. With *CX* the transfer is quantitative. In the case of *CC* a small fraction is retained in the water phase, but since the distribution ratio between water and ether is constant within the reported range, the value of *E* in ether remains proportional to the *CC* concentration of the original solution (Fig. 1). Furthermore, ether extraction renders the determination relatively specific, the red colour formed by phenol with the reagent being very largely ether-insoluble (see also Table 2).

The inclusion of reagent 1 in the reaction mixtures establishes the desired acidity and accelerates the colour reactions. With solutions containing substances precipitable by Millon solution, reagent 1 also serves to remove the interfering substances. In this case the test solution is centrifuged or filtered 1 min. after addition of reagent 1, and reagent 2 is then added to a portion of the cleared solution (see method for urine). Large variations in the concentration of reagent 1 (one-half to twice the indicated amount) are without effect on the intensity of the colour developed with *CX*. Therefore, the partial removal of reagent 1 by precipitation does not perceptibly affect the colour intensity obtained. In pure aqueous solutions, use of reagent 1 is, of course, not strictly essential.

Under the conditions specified, the indicated reaction times suffice to allow the colour intensity to

reach a peak. Longer incubation periods are inconvenient and may entail some fading of the colour. Attempts to hasten the reaction by the use of higher temperatures were unavailing. At 60° the values of *E* were relatively low; at 95° *CX* failed altogether to give a colour with Millon reagent. On the other hand, non-halogenated phenols, as is well known, react most strongly with the reagent when boiled. Use might be made of this differential character of the reaction to determine non-halogenated in the presence of halogenated phenols. Increase in the relative amount of reagent 1 employed considerably increases the amount of water-soluble coloured substance formed with the Millon reagent by non-halogenated phenol.

B. Procedure with biological materials

Proteins, fats, and some inorganic salts give a precipitate with Millon reagent and interfere with its use in colorimetry. However, quantitative separation of halogenated phenols from various interfering substances present in biological material can be effected by extraction with toluene, steam distillation, and precipitation with reagent 1. The use of the two latter procedures is illustrated below.

Determination of *CX* in urine. 2 ml. of urine are diluted with 4 ml. of water and then 2 ml. of reagent 1 are added. After 3 min. the mixture is centrifuged, 4 ml. of the clear supernatant fluid are transferred to a test-tube, and 0.3 ml. of reagent 2 added. The solution is allowed to stand for 30 min. at room temperature, cooled, extracted with 4 ml. of chilled ether, and colorimetry carried out as in the method for aqueous solutions. A standard curve is constructed from results obtained with water solutions of known *CX* content similarly treated. The sensitive range is that covered in the experiment described in Table 1.

Determination of *CC* in urine. 5 ml. of urine are made just acid to litmus, transferred to a long-necked distillation flask, and steam-distilled until 15 ml. of distillate have collected. Excessive foaming is prevented by the addition of a few drops of liquid paraffin. The distillate obtained is treated as in the procedure given for determination in water. A standard curve is prepared from results obtained with aqueous solutions of known content distilled in the same apparatus and treated similarly. The sensitive range is shown in Table 1.

Determination of *CX* in blood. 5 ml. of oxalated blood are deproteinized by mixing with 20 ml. acetone, filtered under suction, and the precipitate washed with 10 ml. acetone and 5 ml. ether. The pooled filtrates are quantitatively transferred to a long-necked distillation flask, joined to a water condenser and held over a water-bath. 0.3 ml. 40% NaOH and, to diminish foaming, a few drops of liquid paraffin are added. The acetone and ether are then driven off slowly by heating the water-bath. Evaporation is discontinued when, the constant flow of distillate having ceased, foaming becomes excessive. The residue of liquid is then rendered acid by addition of 1 ml. 25% (v/v) H₂SO₄, and steam-distilled. When 15 ml. of distillate have collected, the distillation is stopped. The distillate or a suitably diluted portion is used as in the method for

aqueous solutions. A standard curve is prepared by subjecting aqueous solutions of known concentration to similar treatment, using the same apparatus. The sensitive range is shown in Table 1.

The same procedure can be applied to the determination of *CC* in blood.

Determination of *CX* and *CC* in minced tissues. Extraction of *CX* and *CC* is carried out from minced tissue by boiling in acetone under a reflux condenser for 1 hr. Otherwise the procedure follows the method for blood.

distillation procedures is therefore a small and, within the error of estimation, a constant fraction of the total amount originally present. Hence the incomplete recovery on distillation does not invalidate the procedure. It is therefore important, however, to standardize the distillation procedure very accurately.

Specificity of estimation. The method is highly specific for halogenated phenols. The examination

Table 1. Recovery of halogenated phenols from biological materials

Test substance	Medium		mg./100 ml.						
			0	7	11	24	38	57	80
<i>p</i> -Chloro- <i>m</i> -xylenol (<i>CX</i>)	Urine	Added	0	7	11	24	38	57	80
		Found	0	8	11	25	36	57	78
<i>p</i> -Chloro- <i>m</i> -xylenol (<i>CX</i>)	Blood	Added	0	2.0	4.0	4.0	6.0	6.0	—
		Found	0	2.0	3.8	4.0	5.6	5.7	—
<i>p</i> -Chloro- <i>m</i> -cresol (<i>CC</i>)	Urine	Added	0	5	10	15	20	25	—
		Found	0	5	11	15	20	25	—

Results. The analytical findings when halogenated phenols were added in known amount to urine and blood are seen from representative experiments summarized in Table 1. Within the range of the reported experiments the method can be relied upon to give results with a maximum error of about 5%.

Table 2. Influence of phenol on estimation of *p*-chloro-*m*-xylenol (*CX*)

Composition of test mixture		Analytical finding	
<i>CX</i> (mg./100 ml.)	Phenol (mg./100 ml.)	<i>E</i>	Estimated <i>CX</i> (mg./100 ml.)
(a) Solution in water			
1.6	0	0.40	1.6
1.6	4	0.40	1.6
1.6	7	0.41	1.6
1.6	14	0.45	1.8
1.6	28	0.61	2.4
3.2	0	0.84	3.2
3.2	16	0.82	3.1
3.2	32	0.86	3.3
3.2	49	1.14	4.3
0	28	0.10	0.4
0	56	0.20	0.8
0	160	0.70	2.7
(b) Solution in urine			
20	0	0.35	20
20	20	0.35	20
20	40	0.36	21
20	100	0.39	22
20	200	0.41	23
20	400	0.53	30

The absolute recovery of chlorophenol in the methods involving separation by distillation was 93–98% in these and similar experiments on *CX*, and 83–87% for *CC*. The loss of chlorophenol in the

of 1000 urine samples from human subjects, none of whom had received chlorophenol prior to the test, failed to reveal a single urine showing a positive reaction for halogenated phenol. The blood of normal untreated humans is likewise consistently negative.

Tyrosine does not react with Millon reagent under the conditions of the test, nor do phenol and cresol in the concentrations present in urine. The degree of interference with the estimation of *CX* resulting from the presence of unhalogenated phenol is indicated in Table 2. The relative yields of the ether-soluble coloured substance, with *CX* and phenol respectively, are at least 50:1. Interference with *CX* estimation was marked only when the concentration of phenol was over 10 times that of *CX*. In the presence of *CX* there is a measurable increase in the intensity of the colour of the ether-soluble material formed with phenol. The effect is, however, small and is not a serious source of error. In the method used with urine, phenol interferes with the determination of 20 mg./100 ml. only at concentrations greater than 100 mg./100 ml. (Table 2 (b)). The normal physiological level of 'free phenol' in urine, however, is only 5–30 mg./100 ml.

SUMMARY

1. The application of the Millon reaction to the determination of halogenated phenols in the presence of phenol is described.

2. Semi-micro-procedures for the determination of *p*-chloro-*m*-xylenol and *p*-chloro-*m*-cresol in water, urine, blood, and minced tissue are given in detail.

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