SHORT COMMUNICATIONS

The quantitative colorimetric determination of colchicine in aqueous solution, and studies on its application to urine*

The oral administration of colchicine to relieve acute attacks of gout has been practized for many years. Few studies of its metabolism and excretion have been made in human subjects. After intravenous injections of relatively large doses to rats, BRUES[1] has reported the presence of colchicine in their urine, as determined by the chemical technique of BOYLAND AND MAWSON[2]. This technique, however, is not sufficiently sensitive to detect smaller excretion which would be expected in human subjects after a therapeutic dose of from 4 to 6 mg *per diem*. In the present study, improved methods for the extraction, separation and colorimetric estimation of colchicine have been developed. The procedure has yielded good recoveries of colchicine added to water or to extracts of urine. Colchicine added directly to urine was incompletely recovered, suggesting that an alteration in its properties had occurred.

METHODS AND RESULTS

Materials

1. Colchicine** purified according to the procedure of ASHLEY AND HARRIS[3]. Recrystallization from ethyl acetate yielded pale yellow crystals, m.p. 151–152° (uncor.).

2. Colchiceine prepared by the method of SORKIN[4] from colchicine yielding a white crystalline material, m.p. 168–170° (uncor.).

3. Benzene, redistilled.

4. Ethylene dichloride, redistilled.

5. Formamide, C. P., stored in desiccator over concentrated H_2SO_4.

6. Whatman No. 1 and No. 4 paper for chromatography were previously washed for seven days in ethanol and seven days in water by descending chromatography.

Procedures

Colorimetric determination of colchicine. In the existing procedures (BOYLAND AND MAWSON[2]; KING[5]), colchicine is hydrolyzed to yield colchiceine, which can readily be determined by means of the olive green color it gives upon addition of ferric chloride. The absorption spectrum of this complex exhibits maxima at 470 and 630 mμ. In order to minimize the absorption of brown-colored extraneous material in urine

* This research was supported by funds granted by the Lillia Babbitt Hyde Foundation, New York, N.Y.

** Obtained from S. B. Penick & Co., 50 Church Street, New York 7, N.Y.

extracts, we have measured optical densities at 630 rather than at 470 mμ. Conditions of hydrolysis which give almost 100% conversion to colchiceine have been adopted.

Various quantities of colchicine (from 100 to 1000 μg) were dissolved in 1 ml of *N* HCl in 50% ethanol and transferred to 15 mm by 150 mm test tubes. The test tubes were covered with tare bulbs, and placed in a boiling water bath for 30 min. To each tube was then added 0.5 ml of distilled H_2O, 2 ml of *N*/10 aqueous HCl, and 0.01 ml of 10 *M* $FeCl_3$. The resultant solutions are read at 630 mμ in a Coleman Model 6B Spectrophotometer. Table I shows that the resulting optical densities

TABLE I

THE ESTIMATION OF COLCHICINE BY HYDROLYSIS AND REACTION WITH FERRIC CHLORIDE

μg	Optical density*		% conversion***
	colchicine	colchiceine**	
100	0.121	0.123	98.4
200	0.243	0.245	99.2
300	0.360	0.369	97.6
400	0.485	0.490	99.0
500	0.601	—	—
1000	1.23	—	—
			Average: 98.6

* Average of three results each.
** Colchicine is converted to colchiceine with a 92% theoretical yield. The optical density of colchiceine was corrected by using 92% of the density obtained.
*** % conversion is equal to the per cent of the theoretical yield (92%) obtained.

TABLE II

EXTRACTION OF COLCHICINE FROM AQUEOUS SOLUTIONS AT VARIOUS pH'S WITH DIFFERENT SOLVENTS

Solvent	pH	Average % recovered*
Chloroform	7	60
	4	79
	9	72
Benzene	5	35
	9	34
	7	34
Ethyl acetate	5	62
	9	59
	7	62
Ethylene dichloride	5	87
	9	85
	7	94
	7**	88
	7***	81

* Based on three experiments.
** 20 ml portions of solvent.
*** 10 ml portions of solvent.

followed Beer's Law, and that an average 98.6% of the theoretical conversion to colchiceine was obtained.

Extraction of colchicine from water. 40 ml aqueous solutions, containing 1 mg of colchicine, adjusted to specified pH values with HCl or NaOH were extracted three times with 30 ml portions of solvent. The residues from the combined extracts, after evaporation, were assayed for colchicine. Table II shows that extracting with ethylene dichloride at pH 7.0 was the most efficient. A decrease in the ratio of solvent to aqueous medium volume showed a decrease in efficiency of extraction.

Since 1 *N* NaOH removes much of the colored material from the ethylene dichloride extracts of urine, the loss of colchicine by such washes was studied.

A 40 ml aqueous solution containing 1 mg colchicine at pH 7 was extracted three times with 30 ml volumes of ethylene dichloride. The combined extracts were washed twice with 10 ml of *N* NaOH and once with 10 ml ethylene dichloride. The total combined ethylene dichloride extracts were assayed for colchicine. It may be seen (Table III) that a loss of about 4% occurred as a result of the NaOH washes.

TABLE III

EFFECT OF AQUEOUS SODIUM HYDROXIDE WASH ON ETHYLENE CHLORIDE EXTRACTS OF COLCHICINE SOLUTIONS

Sample	*% colchicine recovered*
1	88.8
2	89.7
3	90.5
Average:	89.7

TABLE IV

RECOVERY OF COLCHICINE IN THE EFFLUENTS OF 36 cm LONG CHROMATOGRAMS DEVELOPED BY THE ZAFFARONI TECHNIQUE[6] USING A BENZENE–FORMAMIDE SYSTEM

The effluents were evaporated and determined for colchicine.

Chromatogram No.	*Whatman paper No.*	*Days collected**	*Colchicine chromatographed µg*	*Colchicine recovered µg*	*Colchicine recovered %*
1	1	2–12	600	546	91
2	1	2–12	600	603	101
3	1	2–12	600	611	102
4	1	2–12	400	367	92
5	1	2–12	400	384	96
6	1	2–12	400	373	93
7	4	1– 7	1000	968	97
8	4	1– 7	1000	984	98
9	4	1– 7	1000	952	95
10	4	1– 7	1000	936	94
				Average recovery:	96 ± 3%

* Days of chromatographic development during which effluent was collected.

When colchicine was added to such washed extracts of urine, only 50–70% recovery was obtained after hydrolysis and colorimetry.

Paper chromatographic separation

In the belief that recoveries of colchicine from urine extracts could be improved by separation of the remaining pigments and other neutral materials, chromatographic fractionation of colchicine was undertaken. It was found that colchicine could be effectively chromatographed in the benzene–formamide system developed by BURTON, ZAFFARONI AND KEUTMANN[6], on either Whatman No. 1 or No. 4 paper (Table IV). On the No. 1 paper, colchicine has a mobility of 0.4 cm per hour, which enabled it to be adequately separated from polar urine material left at the origin and a low-polarity pigment fraction that moved with the solvent front. The presence of colchicine on the paper could be shown by testing with concentrated HCl, whereupon a yellow-green band was obtained. However, the colchicine could not be quantitatively eluted from the paper with a variety of solvents (*i.e.*, ethanol, methanol, chloroform and ethyl acetate) and techniques (*i.e.*, macerating and pulverizing the papers in the hot solvents), all of which gave low recoveries. Recourse was therefore had to longer periods of chromatography, combined with a shorter strip of the faster No. 4 paper, in hopes that the colchicine would be found in the effluent. In the final procedure, the first effluents, containing the fast moving pigments, were discarded. Collections were then made after various periods of chromatography. Table V shows that good recoveries of colchicine added to urine extracts were obtained.

Application of techniques to urine specimens. On the basis of the above experiments, a method for the extraction of colchicine from urine was proposed. A 1 l specimen of normal urine containing colchicine, adjusted to pH 7, was extracted three times with 750 ml of ethylene dichloride. The combined extracts were washed twice with 225 ml

TABLE V

RECOVERY OF 1 mg OF COLCHICINE AFTER ADDITION TO EXTRACTS OF 1000 ml OF NORMAL URINE

Effluents from the chromatogram in a benzene–formamide system were collected after the low polarity urine pigments had visibly descended from the paper*, and were determined for colchicine.

1000 ml normal urine extract from urine pool No.	*Whatman paper No.*	*Length of paper (cm)*	*No. of days effluent collected*	*Average % colchicine recovered***
1	1	36	10	80
2	1	36	10	77
3	1	36	10	80
1	1	36	20	100
2	1	36	20	99
3	1	36	20	91
4	1	16	10	87
5	1	10	10	87
6	4	5	7	87

* A 36 cm Whatman No. 1 paper about 1 h, and a Whatman No. 4 paper about 20 min.
** Based upon two experiments.

of *N* NaOH and once with the same volume of water. The combined washes were backwashed with 500 ml of ethylene dichloride. This was combined with the previous ethylene dichloride extract and distilled on a steam bath *in vacuo*. The residue was chromatographed as previously described, on a 5 cm Whatman No. 4 paper. The seven day effluent was assayed for colchicine by evaporation, hydrolysis and reaction with ferric chloride.

When 1 mg of colchicine was added to two separate urine specimens and processed in the above manner, recoveries of only 6 and 9% were obtained. Since colchicine had been efficiently extracted from water, and recovered from extracts of urine previously prepared by the identical procedure, this failure was not readily explained. More vigorous extraction procedures were tried, in hopes of overcoming an apparent lower extractability of colchicine from urine than from water. However, neither *n*-butanol nor continuous ether extractions altered the final recoveries substantially. It must be concluded that an unknown alteration in the properties of colchicine results when it is added to urine.

DISCUSSION

The data presented show that the analytical techniques employed to extract colchicine from urine are valid. The poor, final results obtained must be attributed to some modification of the partition relations of colchicine. This hypothesis would explain the contradictory results obtained by BRUES[1] and LETTRÉ[7].

BRUES, using rat urines, estimated colchicine by the method of BOYLAND AND MAWSON[2]. This entailed hydrolysis of the urine in *N*/10 HCl at 100° for one hour and estimation of the colchicine as colchiceine by extraction with chloroform and reaction with ferric chloride. LETTRÉ, using extracts of unhydrolyzed urines, found an absence of the mitotic inhibiting effect on chicken heart fibroblasts associated with colchicine. The difference between their results may possibly be related to the additional hydrolysis procedure. It is well known that various conjugates are decomposed by acid hydrolysis. Analogously, this could explain BRUES' recovery and LETTRÉ's lack of recovery of colchicine from urine.

In the present study hydrolysis before extraction was not performed, since the resulting extract could not be washed with NaOH without removing the phenolic-like colchiceine, as well as extraneous matter and pigments. Further studies are necessary before the phenomenon observed can be understood.

The author is grateful to EDWARD F. HARTUNG, M.D., HILDEGARD WILSON, Ph.D., and MARGARET NEIL, for encouragement, advice and technical suggestions.

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Received September 11th, 1958

Ein neues papierchromatographisches Verfahren

Sowohl absteigende, aufsteigende, als auch horizontale Trennungen finden ihre Grenzleistung in der Steighöhe, bzw. Laufstreckenlänge.

Auf Grund einer einfachen Überlegung und der sich aus dieser ergebenden Technik ist es möglich, den Wanderweg eines Gemisches beliebig zu verlängern und dadurch die Trennung seiner Komponenten im Vergleich zu den bisher möglichen Ergebnissen um 100% zu verbessern.

Die Versuche wurden aus gegebenem Anlass mit Aminosäuregemischen und Fruchtsäuregemischen vorgenommen. Die Trennungen erfolgten nach der horizontalen Methode unter Benutzung eines Rundfilterentwicklungsgerätes.

Technik

Der Durchmesser des Rundgerätes betrug 30 cm, das Papier wurde in Rechteckform von 30 cm Breite und 45–60 cm Länge geschnitten. Das Laufmittel—in unserem Fall *n*-Butanol–Ameisensäure–Wasser—kam in ein Schälchen innerhalb des Gerätes.

Den Laufmitteltransport besorgte ein Röllchen (Docht) aus dem gleichen wie zum Chromatogramm verwendeten Papier. Es wurde aus etwa 10 bis zu 30 cm langen und je nach gewünschter Länge des Tauchdochtes 4 bis 5 cm breiten Papierstreifen gerollt. Die Dochtstärke betrug bis zu 1 cm. Das zu untersuchende Gemisch hatte seinen Startpunkt in der mittleren Längsachse des Chromatogramms, etwa 2–3 cm vom Saugdocht entfernt. Dieser sitzt beim Anlaufen in der Mitte des Gefässes, ist also bei dem gegebenen Durchmesser des Entwicklungsgerätes dessen Radius entsprechend 15 cm vom Schmalrand des Papierrechtecks entfernt und genau in dessen Mittelachse. Er taucht dort in das mit dem Laufmittel gefüllte Schälchen. Sobald die Laufmittelfront den kreisförmigen Gefässrand erreicht hat, wird das Schälchen nachgefüllt und aus der Gerätmitte an dessen Peripherie in "Randdochtstellung" geschoben. Der Papierbogen wird in Richtung seiner Längsachse mitverschoben und mit dem gleichen Docht nunmehr aus Randdochtstellung weitergetrennt. Durch diesen Vorgang ragt ein Teil des feuchten Papierbogens aus dem Gerät heraus und wird abgeschnitten. Gleichzeitig ist ein entsprechendes Stück des noch nicht durchlaufenen, trockenen Papierrechtecks auf der gegenüberliegenden Geräteseite in dessen Inneres gezogen worden. Es ist darauf zu achten, dass Gerät und Deckel breite,