CHROM. 5418

AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

V. THE QUANTITATIVE DETERMINATION OF UREA IN URINE

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(Received March 30th, 1971)

SUMMARY

A simple, rapid method is described for the accurate quantitative determination of urea in undesalted urine using uni-dimensional thin-layer chromatography (HEATHCOTE AND HAWORTH). The method is based on the formation of a stable coloured complex between urea and Ehrlich's reagent which is then estimated quantitatively. Several urine samples have been examined by this method and the results agree favourably with those obtained by two other standard procedures.

INTRODUCTION

The estimation of urea in urine is one of the most important determinations in the clinical laboratory, and several different procedures[1–5] have been employed. Of these, two procedures are used principally. The first involves the measurement of the ammonia released by the action of the specific enzyme urease[3], but the operation is time-consuming and is not readily adapted to serial analysis. Another common procedure employs an auto-analyzer to determine the coloured complex produced with diacetyl monoxime or other suitable reagent[5,6]. This is a rapid method but requires appreciable operator time and also monopolises expensive instrumentation for what should be a straight forward analysis.

Thin-layer chromatography (TLC) followed by densitometry has already provided a rapid and reliable method for the estimation of amino acids and related compounds present in biological fluids[7,8] and such procedures require little operator or instrument time.

We have now developed a simple, one-dimensional chromatographic procedure for the determination of urea in urine. A stable and reproducible colour is formed with Ehrlich's reagent[9] and this permits the plotting of a standard graph. This TLC method has been applied to urine samples, and the results obtained agree well with those obtained by the automatic procedure and also with those derived from the urease method.

EXPERIMENTAL

Apparatus

The thin-layer equipment used was supplied by Shandon*. The automatic recording and integrating double beam reflectance densitometer, "Chromoscan", with thin-layer attachment was used for the quantitative evaluation of the chromatograms**. The results obtained by densitometry following chromatography were compared with those obtained by the automatic procedure using the Technicon instrument***.

Materials and methods

Preparation of thin layers of cellulose. Cellulose powder [MN300 (without binder)]§ was spread over glass plates (20 × 20 cm) at a thickness of 400 µ using the procedure described previously[10]. It was not found necessary to wash the cellulose because the coloured impurities present in the cellulose do not interfere with the separation of the urea.

Standard solutions. A stock solution of urea (0.05 *M*) was prepared using aqueous 2-propanol (10%) as solvent. The stock solution was diluted stepwise (to 0.005 *M*) as required and, when not in use, solutions were kept in the refrigerator.

Application of samples to thin layers. In order to keep the amount of solvent required to a minimum and to maintain a standard procedure, chromatography was always parallel to the margin of unspread plate left by the Shandon equipment. A line was marked 1.5 cm up from the lower edge of the layer using a soft lead pencil. The starting points were then positioned 1.5 cm from each other along this line. In order to reduce errors due to edge effect to a minimum, a margin (1.5 cm) was left at each edge of the layer. Care was taken not to disturb the surface of the layer in order to prevent distortion of the spots during subsequent chromatographic development. The limit (13 cm from the origin) to which the solvent front was to be allowed to rise was then marked at the edge of the layer. Each urine sample (1 µl) was applied to its marked origin. It was found possible to place a total of ten urine samples on the one 20 × 20 cm plate.

Development. The plates containing the samples were placed in chromatographic tanks containing 50 ml of the solvent 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25) (ref. 10). The atmosphere in each tank had been pre-saturated with the solvent before use as described by HEATHCOTE AND HAWORTH[11]. After the ascending solvent front had travelled 13 cm from the origin (2.5 h), the plate was removed and dried in a stream of air for 15 min to remove excess hydrogen chloride. The plate was then heated at 60° for 15 min or until free from organic solvent (no detectable odour).

Detection of urea. The reagent used for the detection and quantitative determination of urea on thin layers of cellulose was Ehrlich's reagent[9]. It consists of *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid (10% w/v). This was added to four times its volume of propanone. The reagent is unstable and was always

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§Macherey, Nagel & Co. Ltd., Agents Camlab (Glass) Ltd., Cambridge.

prepared immediately prior to use. The spray reagent was applied to the thin-layer plate using a Shandon atomiser at a distance of about 30 cm until the plates just appeared translucent, excess reagent being avoided to preclude any diffusion of the spots. With all urine samples examined to date, the yellow coloured spot corresponding to urea appeared on the layer immediately on spraying. It was therefore unnecessary to spray the whole layer with the reagent but only a narrow band corresponding to the position of the urea.

Reproducible results were not obtained by allowing the sprayed plates simply to stand at room temperature. Accordingly, a study was made of the time taken for the colour to develop at different temperatures and also of the effect of storing the plates, after staining, on this colour. Several thin layer plates were spotted with the same range of amounts of urea. They were then chromatographed and sprayed with the reagent. The colour was developed in the dark at differing temperatures (45°, 60° and 80°) for varying periods of time (15, 30 and 45 min), they were allowed to cool, and read on the densitometer at intervals of 0, 2, 4, 6 and 24 h. Between readings the plates were kept in the dark.

Densitometry. Since the urea complex gave a yellow colour, a light filter (405 nm) complementary to this colour was used to give maximum absorbance. The aperture used throughout was the 1005 (10 × 0.5 mm) slit, and the gear ratio was 1:2. As recommended by DALLAS[13] two thicknesses of Whatman No. 3MM filter paper were placed immediately behind the plate. The stained spots were scanned across the layer at right angles to the direction of chromatography. In this way a single adjustment

TABLE I

COLOUR DEVELOPMENT OF UREA AT 40° ON THIN LAYERS OF CELLULOSE AFTER STAINING WITH EHRLICH'S REAGENT

Storage time (h)	*Urea (µg)*										*Mean C.V. (%)*
	3.0	*6.0*	*9.0*	*12.0*	*15.0*	*18.0*	*21.0*	*24.0*	*27.0*	*30.0*	
	Mean area under densitometric curve (mm²)										
15 min development time											
0	—	—	—	—	—	—	—	—	—	—	—
2	312	621	785	923	1088	1210	1371	1431	1541	1692	10.3
4	567	780	1091	1233	1391	1468	1571	1637	1701	1785	10.7
6	532	777	1092	1301	1397	1453	1584	1634	1709	1779	10.0
24	559	893	1123	1375	1512	1710	1901	2101	2234	2420	9.1
30 min development time											
0	121	198	283	365	451	541	710	1132	1371	1634	51.1
2	396	544	750	863	993	1161	1312	1517	1628	1836	21.1
4	532	763	895	1341	1497	1647	1791	1943	2101	2200	8.2
6	588	960	1231	1447	1630	1783	1901	2150	2317	2420	6.3
24	576	920	1217	1471	1652	1817	1953	2117	2271	2400	4.9
45 min development time											
0	422	698	791	1201	1331	1575	1678	1785	1924	2170	14.0
2	448	783	1103	1353	1529	1731	1812	2001	2159	2317	9.4
4	564	920	1247	1459	1631	1800	1947	2093	2255	2464	6.3
6	576	917	1233	1451	1682	1809	1963	2117	2279	2437	4.8
24	611	921	1281	1521	1697	1883	2017	2091	2312	2530	3.5

TABLE II

COLOUR DEVELOPMENT OF UREA AT 60° ON THIN LAYERS OF CELLULOSE AFTER STAINING WITH EHRLICH'S REAGENT

Storage time (h)	*Urea (μg)*										*Mean C.V. (%)*
	3.0	*6.0*	*9.0*	*12.0*	*15.0*	*18.0*	*21.0*	*24.0*	*27.0*	*30.0*	
	Mean area under densitometric curve (mm²)										
15 min development time											
0	407	485	573	663	761	825	1070	1147	1317	1512	43.1
2	432	541	682	821	933	1017	1222	1371	1551	1720	27.7
4	432	650	820	988	1132	1241	1413	1551	1637	1805	23.0
6	494	707	931	1133	1291	1388	1545	1673	1817	1935	21.2
24	533	821	1131	1361	1562	1681	1870	2041	2158	2300	9.0
30 min development time											
0	455	595	743	903	1053	1213	1379	1676	1900	2280	8.1
2	546	698	840	1021	1180	1332	1545	1773	2107	2418	5.4
4	585	779	1010	1209	1431	1572	1801	2011	2292	2540	5.3
6	585	818	1211	1432	1621	1841	2041	2251	2403	2583	6.2
24	620	1001	1307	1532	1741	1913	2171	2257	2421	2535	4.9
45 min development time											
0	630	952	1188	1371	1519	1701	1852	1988	2107	2228	3.3
2	644	1018	1247	1457	1613	1807	1956	2101	2209	2392	3.7
4	621	940	1180	1381	1539	1721	1867	2031	2140	2275	2.9
6	633	959	1211	1389	1575	1719	1848	2017	2104	2223	3.5
24	720	1031	1281	1492	1631	1801	1941	2141	2213	2394	4.1

TABLE III

COLOUR DEVELOPMENT OF UREA AT 80° ON THIN LAYERS OF CELLULOSE AFTER STAINING WITH EHRLICH'S REAGENT

Storage time (h)	*Urea (μg)*										*Mean C.V. (%)*
	3.0	*6.0*	*9.0*	*12.0*	*15.0*	*18.0*	*21.0*	*24.0*	*27.0*	*30.0*	
	Mean area under densitometric curve (mm²)										
15 min development time											
0	275	532	872	1037	1250	1339	1551	1601	1750	2100	9.2
2	221	621	864	1026	1216	1387	1553	1619	1777	2266	8.3
4	390	650	921	1071	1228	1400	1567	1676	1783	2314	7.6
6	429	671	997	1108	1233	1471	1662	1831	1823	2300	7.7
24	416	632	921	1066	1220	1421	1548	1701	1799	2354	5.4
30 min development time											
0	516	901	1243	1411	1613	1757	1921	2001	2047	2318	5.5
2	540	887	1347	1528	1721	1808	2001	2103	2191	2486	4.4
4	559	873	1149	1347	1528	1763	1912	2101	2209	2457	10.0
6	551	865	1171	1348	1571	1732	1881	2051	2217	2398	5.5
24	523	833	1192	1407	1549	1763	1892	2076	2103	2300	5.5
45 min development time											
0	492	731	1032	1250	1420	1598	1772	1931	2117	2310	3.5
2	494	762	1071	1239	1433	1631	1794	1951	2101	2268	4.2
4	559	892	1221	1401	1550	1701	1889	2091	2317	2671	3.5
6	559	921	1237	1397	1499	1711	1907	2033	2404	2691	3.3
24	525	822	1131	1271	1471	1647	1801	1949	2101	2352	2.7

to the instrument usually sufficed. The area under the densitometric curve was obtained from the product of the altitude and the width of the curve at half the maximum height.

RESULTS AND DISCUSSION

Optimum conditions for staining reagent

A comparative study of Tables I, II and III shows that colour development either at 80° or at 60° for a time of 45 min produced values with a coefficient of variation of about 3% and the values did not alter subsequently with time. Accordingly a temperature of 60° and a time of 45 min were chosen as the conditions for the colour development in the determination of urea in urine.

TABLE IV

$R_F \times 100$ VALUES OF UREA AND RELATED EHRLICH POSITIVE SUBSTANCES AFTER CHROMATOGRAPHY ON THIN LAYERS OF CELLULOSE USING THE SOLVENT SYSTEM 2-PROPANOL, BUTANONE, 1 *N* HYDROCHLORIC ACID (60, 15, 25, v/v)

Compound	$R_F \times 100$	*Compound*	$R_F \times 100$
Urea	68	Allantoin	49
Indican	89	*p*-Aminohippuric acid	82
5-Hydroxytryptophan	43	Anthranilic acid	97
3-Indolyllactic acid	96	3-Indolylacrylic acid	97
Tryptamine	73	Kynurenic acid	91
3-Indolylacetic acid	98	Kynurenine	38
Hippuric acid	71	Citrulline	34
Tryptophan	71	Indole	97
N,N'-Dimethyltryptamine	87	Xanthurenic acid	91
3-Hydroxykynurenine	30	Thiourea	71

Specificity of method

It will be seen from Table IV that, of the compounds which are present in urine, and which react with Ehrlich's reagent, only hippuric acid, thiourea and tryptophan have R_F values which are likely to interfere with the determination of urea by this procedure. However, the amounts of these compounds which are present in urine are very small compared with the amount of urea and so any interference would be negligible except possibly in certain pathological conditions, *e.g.* Hartnup disease.

Preparation of standard curves

After chromatography of a range of amounts of urea (3.0–30.0 μg) each plate was stained with reagent and the developed spots were scanned with the densitometer. A linear relationship was obtained (Fig. 1) when the urea under the densitometric curve was plotted against the square root of the amount of urea as described previously for amino acids[11]. From Fig. 1 the slope (406) and intercept (−31) were calculated.

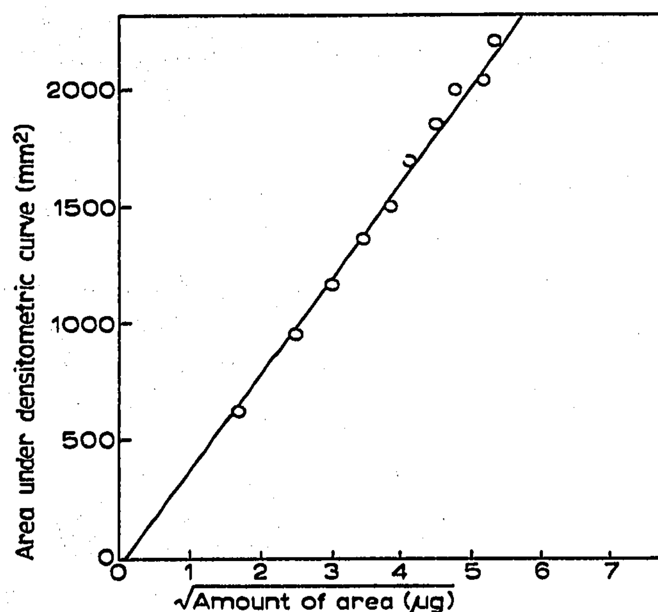

Fig. 1. Colour yield of urea with Ehrlich's reagent.

Determination of urea in urine

The equation of the line was used to determine the amount of urea in urine. The results obtained by the densitometric procedure were compared with those obtained by the automatic procedure using the Technicon instrument[6] and also with the values obtained using the urease[14] procedure. The results obtained for ten different urine samples are given in Table V, from which it will be seen that the TLC procedure gives results which agree well with those obtained using the automatic procedure. Adding known amounts of urea to urine gave recoveries which averaged 100.6% by the TLC procedure (Table VI) while the variation in repeat analyses of the same sample was less than 3%.

TABLE V

UREA CONCENTRATION IN URINE

Comparison of different methods.

Urine sample	*Present study (TLC)*	*Automatic analyser*[6]	*Urease*[14]
	Urea concentration (mg/100 ml)		
1	900	920	966
2	730	760	783
3	625	600	652
4	911	920	989
5	1124	1160	1219
6	2135	2160	2180
7	1122	1180	1212
8	943	950	988
9	1502	1510	1584
10	1219	1240	1302

TABLE VI

PERCENTAGE RECOVERY FROM URINE OF ADDED UREA USING TLC

Urea in urine sample (mg/ml)	*Urea added (mg)*	*Total urea expected (mg/ml)*	*Total urea found by TLC (mg/ml)*	*Percentage recovery*
5.38	1.0	6.38	6.50	102
6.37	1.0	7.37	7.31	99
2.97	2.0	4.97	4.81	96
0.72	1.5	2.22	2.27	102
4.2	1.5	5.7	5.85	103
1.05	2.0	3.05	3.19	104
2.48	0.5	2.98	3.09	104
1.05	0.3	1.35	1.30	96
4.30	0.1	4.4	4.37	99

Despite the essentially manual nature of the operation, large numbers of samples may be analysed, quickly, simply and accurately using relatively inexpensive equipment.

REFERENCES

1 L. HORN, *Scand. J. Clin. Lab. Invest.*, 3 (1951) 157.
2 A. L. CHANEY AND E. P. MARBACH, *Clin. Chim. Acta*, 8 (1962) 131.
3 I. HOLM-JENSEN, *Scand. J. Clin. Lab. Invest.*, 13 (1961) 301.
4 J. COTTE, C. COLLOMBEL, L. PADIS AND G. CAILLOT, *Ann. Biol. Clin. (Paris)*, 25 (1967) 155.
5 W. H. MARSH, B. FINGERHUT AND H. MILLER, *Clin. Chem.*, 11 (1965) 624.
6 Technicon N. Methodology file N-1c.
7 J. G. HEATHCOTE, D. M. DAVIES, C. HAWORTH AND R. W. A. OLIVER, *J. Chromatogr.*, 55 (1971) 377.
8 E. J. SHELLARD AND M. Z. ALAM, *J. Chromatogr.*, 33 (1968) 347.
9 I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, Heinemann, London, 1960.
10 C. HAWORTH AND J. G. HEATHCOTE, *J. Chromatogr.*, 41 (1969) 380.
11 J. G. HEATHCOTE AND C. HAWORTH *J. Chromatogr.*, 43 (1969) 84.
12 J. G. HEATHCOTE, R. J. WASHINGTON, C. HAWORTH AND S. BELL, *J. Chromatogr.*, 51 (1970) 267.
13 M. S. J. DALLAS, *J. Chromatogr.*, 33 (1969) 337.
14 D. D. VAN SLYKE AND G. E. CULLEN, *J. Biol. Chem.*, 19 (1914) 211.