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CONTINUOUS THIN-LAYER CHROMATOGRAPHY OF SUGARS OF CLINICAL INTEREST IN SAMPLES OF URINE IMPREGNATED ON PAPER

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SUMMARY

A procedure for the analysis of sugars of clinical interest in samples of urine impregnated on Whatman 3MM paper is described. The sugars are eluted from the sample and spotted directly on to the application zone of concentration-zone silica gel plates, followed by continuous development. The optimal composition of the eluent and developing solvent and the optimal development distance were established, together with the development time and its influence on the R_F values. The locating reaction is based on the reduction of vanadium(V) to vanadium(IV) in acidic medium.

INTRODUCTION

Our interest in the identification and assessment of sugars in urine samples impregnated on chromatographic paper stems from our laboratory's participation in a screening programme to detect inborn errors of metabolism. Urine samples are taken from newborn infants, impregnated on Whatman 3MM paper, dried and then posted to our laboratory.

We have developed our own spot test based on the reduction of vanadium(V) to vanadium(IV)[1], which facilitates the detection of the presence of reductants in the samples. Of the other spot tests previously reported, one is based on the reduction of a bismuth salt[2] and the other uses the reagent Anthrone[3]. The detection limits of these previous tests are 0.1 % (100 mg per 100 ml) for less reducing sugars (galactose), and their sensitivity is sufficient for the detection of pathological levels; for more reducing sugars (fructose) the sensitivity is even greater.

For the identification and assessment of sugars in those samples which gave positive results in the spot test, we developed a procedure for direct elution of the sugars from the urine sample to the application zone of a silica gel plate with a concentration zone[1]. Two runs were carried out in ethyl acetate–pyridine–acetic acid–water (60:30:5:15) as reported by Menzies and Mount[4]. The locating reaction was made with the vanadium(V) reagent as proposed by Haldorsen[5]. In all previous procedures using silica gel thin-layer chromatography (TLC) for the determination of sugars in urine samples of clinical interest[2,4,6–8] the urine samples needed to be in liquid form and pre-treatment of the sample was almost always required.

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In this paper the application of continuous TLC (also known as continuous flow, continuous elution and continuous development)[9–18] to the investigation of sugars of clinical interest in the above-mentioned samples is reported.

It has been established[9] that a decrease in solvent strength leads, with few exceptions, to an increase in selectivity in TLC. However, the difficulty of putting this into practice is that the R_F values decrease exponentially with a decrease in solvent strength. This difficulty can be overcome by carrying out a short development with continuous chromatography. These separations need no more time than normal but improve the detection of the spots.

Perry[9] made a detailed study of the influence of solvent strength on selectivity in continuous chromatography. Gocan[17] investigated the resolution of this technique. Various devices have been designed for carrying out the operation[9–16] and we describe one below.

EXPERIMENTAL

Standard solutions

These were prepared from urine free of reducing substances in concentrations ranging from 500 to 50 mg per 100 ml for each of stachyose, raffinose, lactose, lactulose, sucrose, galactose, glucose, fructose, arabinose, xylose, ribose, ribulose, maltose, 3-O-methylglucose, xylulose and mannose. Mixtures of these sugars were also prepared, as follows: (ketoses) raffinose, lactulose, saccharose and fructose in concentrations from 50 to 500 mg per 100 ml of each; (aldoses) lactose, galactose, glucose, arabinose, xylose, ribose and 3-O-methylglucose in the same concentration range. Also, mixtures of these two groups in concentrations from 50 to 250 mg per 100 ml of each sugar were prepared.

These solutions were dropped on to pieces of Whatman 3MM paper, which were then dried in air.

Eluents and solvents

The eluent used for the direct application of the sugar contained in the paper to the concentration zone of the plate was isopropanol–methyl ethyl ketone–25% ammonia solution (15:20:8). The developing solvent was ethyl acetate–pyridine–acetic acid–water (60:30:5:10).

Locating reaction

The spots were revealed with 0.1 *M* ammonium monovanadate in 1 *M* sulphuric acid[5].

Materials

Silica gel 60 TLC plates (without fluorescent indicator) with a concentration zone (11845; Merck, Darmstadt, G.F.R.) were used.

The apparatus used for continuous TLC (Fig. 1) consisted of a conventional chromatographic tank with a modified lid cut longitudinally into three sections. Into the 2-cm wide central section of the lid, two holes, 7 mm in diameter and 11 cm apart, are drilled equidistant from its edges. To allow the insertion of a pipette to refill the vessel containing the developing solvent, a further hole, 11 mm in diameter, is drilled

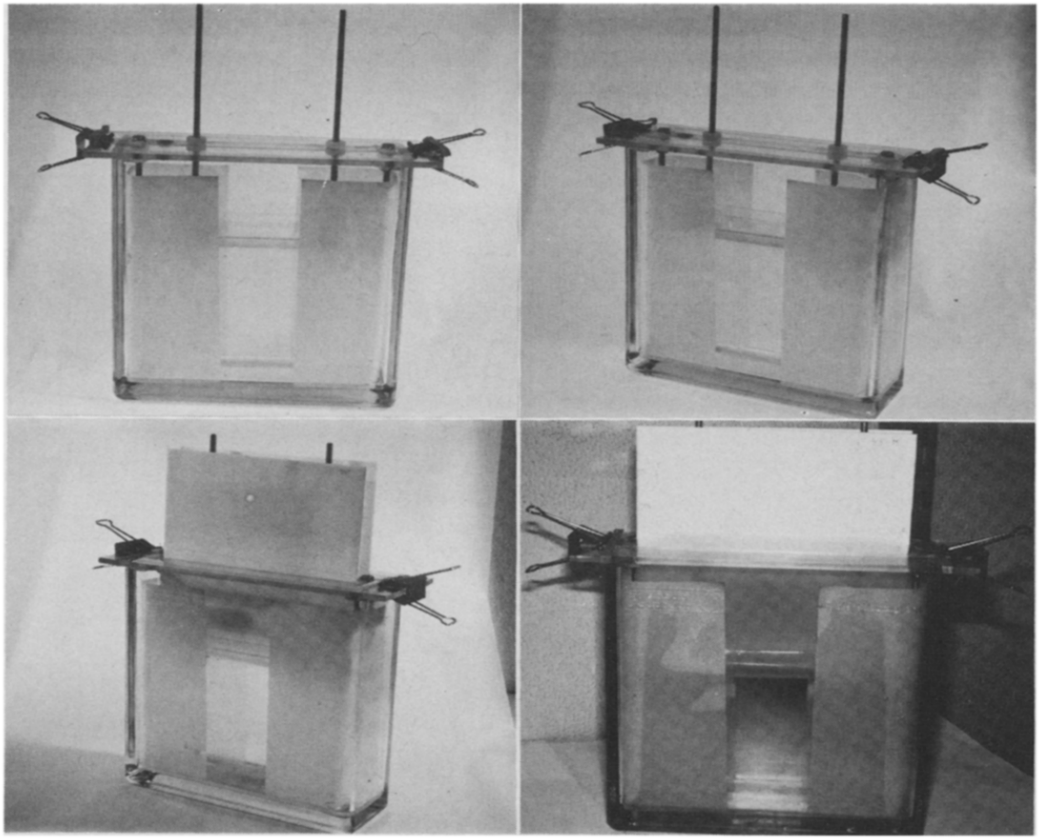


Fig. 1. Apparatus used for continuous TLC.

and fitted with a cap. Wormed screws are introduced into both 7-mm holes and a glass vessel (21 × 4.5 × 2.5 cm deep) is attached to the end of the screws; this vessel contains the developing solvent. The wormed screws are attached to the central portion of the lid by means of two nuts, one below and one above the lid, and by tightening these the lid can be hermetically sealed. Two glass cross-pieces are screwed underneath on to the edges of this central portion of the lid and prevent movement when the lid is in position. The device enables the vessel containing the developing solvent to be moved vertically in order to vary the distance from the solvent front. In this apparatus, one or two plates may be inserted on either side of the central portion of the lid, closing the tank by pressing the two lateral portions of the lid against the plates. The assembly is supported by two pegs, and two elastic bands are used to hold the two lateral sections of the lid against the plates and the central section. The edges of the lid not covering the plates are sealed with silicone grease, the edges of the tank having previously been coated with similar grease to effect a hermetic seal.

Procedure

Sample application and elution. Discs of 4 mm diameter are cut from the urine-impregnated paper with a paper punch, and are placed in the concentration zone of a

chromatographic plate, forming a sandwich as shown in Fig. 2. This is placed for 3–4 min in a chromatographic tank containing the eluent. The sample strip is immediately removed and the application zone of the plate is dried with a cold air blower. The plate is then dipped in the development tank of a continuous chromatograph with two strips of filter-paper on each side and the developing solvent in the bottom, in order to achieve the appropriate saturation.

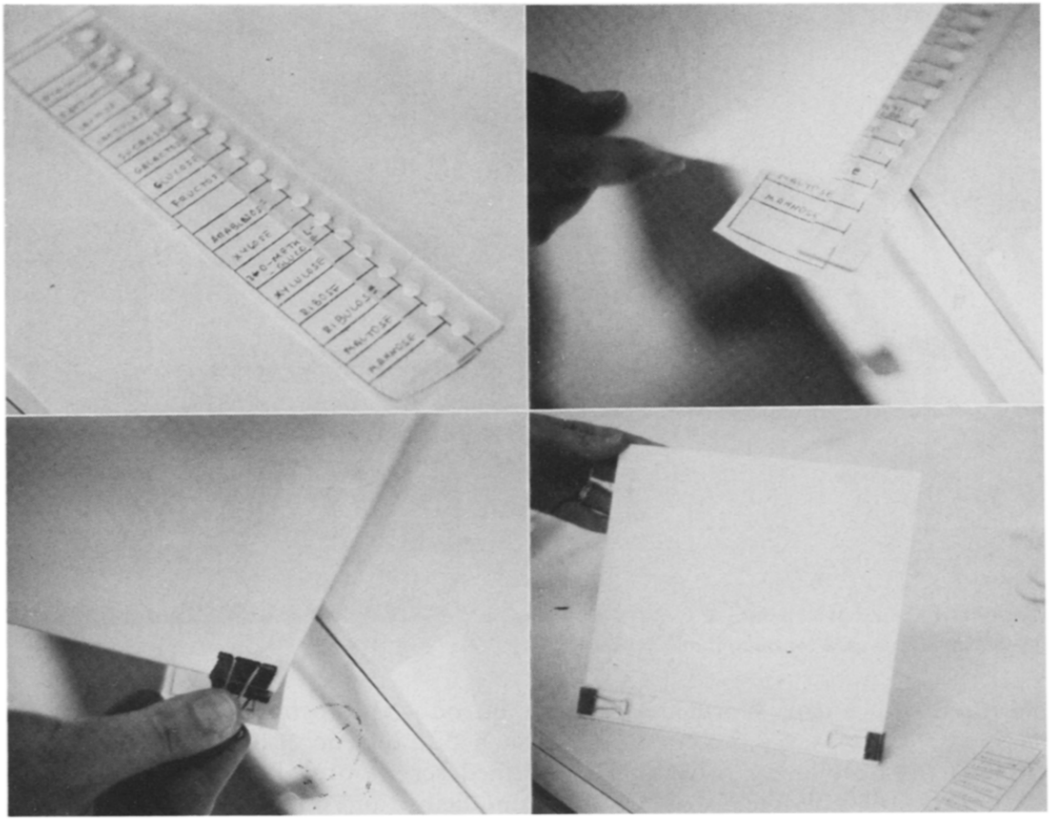


Fig. 2. Sample application. A glass strip (20 × 2 cm) is aligned along the upper edge of the sample identification index. Using forceps, discs are placed on the upper surface. At the extreme edges non-impregnated paper discs are placed. The whole is then placed on the application zone of a silica gel plate with concentration zone. Pressure clips (Foldback 1411) are fixed on the non-impregnated paper discs to avoid arching of the glass strip.

Development. Only one development is carried out, with an evaporation time which is measured from the moment the solvent front reaches the lid of the tank. The chromatographic plate is then dried with a hot air blower for 10 min. We tried various development distances and different evaporation times, noting their influence on both the R_F values and the quality of the chromatograms obtained.

Locating reaction. Once the development is finished, the plate is sprayed with a locating reaction and then placed in an oven at 120°C for 5 min. On removal, the resulting chromatogram shows blue stains on a yellow background which fades with time, as shown in Fig. 3.

RESULTS AND DISCUSSION

Tables I and II show the R_F values for different development distances and evaporation times. The time taken from the start of the development for the solvent front to reach the line of emergence was 45 min at a development distance of 6 cm, 55 min at 7.5 cm and 60 min at 9 cm.

After systematic investigations, the optimal type of plate, method of applying the sample, composition of the eluent and proportions of the developing solvent were established. Studies using plates with and without concentration zones indicated that the former were better, as has been demonstrated previously[1].

When applying the sample, we removed all adherent substances in order to prevent interferences due to their presence to the plate during the elution stage. It is for this reason that the sample is held against the plate only by pressure, as shown in Fig. 2.

The eluent was selected after having tried others in which isopropanol was replaced with *tert.*-butanol and benzyl alcohol. In all instances, various proportions of the components were used.

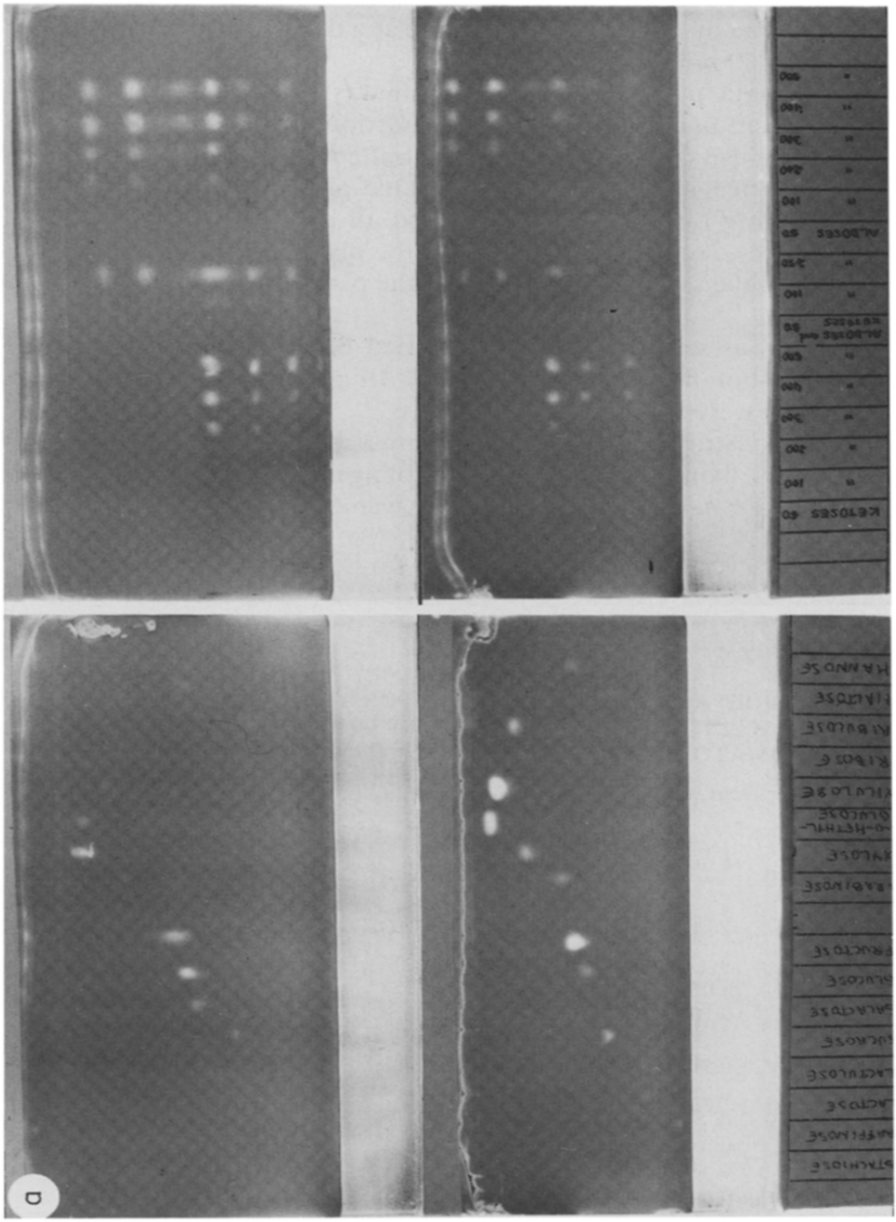
It was demonstrated that the eluent must be prepared immediately before elution is to be carried out, owing to problems of ageing that appear after the locating reaction on the plate, taking the form of transverse bands that interfere with some sugars.

TABLE I

R_F VALUES MEASURED AS A FUNCTION OF EVAPORATION TIME ON PLATES ON WHICH INDIVIDUAL STANDARD SOLUTIONS OF SUGARS IN CONCENTRATIONS OF 500 mg PER 100 ml WERE CHROMATOGRAPHED

Development distance: 6 cm (measured from the separation line of the concentration zone).

Sugar	*Evaporation time (min)*				
	5	*10*	*15*	*20*	*25*
Stachyose	–	–	–	–	–
Raffinose	0.03	–	0.07	–	0.03
Lactose	0.10	0.08	0.19	0.06	0.14
Lactulose	0.10	0.08	0.19	0.10	0.15
Sucrose	0.21	0.22	0.33	0.19	0.33
Galactose	0.21	0.21	0.30	0.18	0.34
Glucose	0.29	0.32	0.42	0.42	0.47
Fructose	0.37	0.40	0.46	0.47	0.53
Arabinose	0.43	0.44	0.55	0.55	0.62
Xylose	0.64	0.63	0.72	0.78	0.80
3-O-Methylglucose	0.88	0.82	0.89	0.99	0.97
Xylulose	0.85	0.79	0.89	0.95	0.95
Ribose	0.71	0.65	0.75	0.80	0.83
Ribulose	0.76	0.69	0.79	0.86	0.86
Maltose	0.18	0.15	0.27	0.25	0.31
Mannose	0.39	0.38	0.52	0.52	0.58



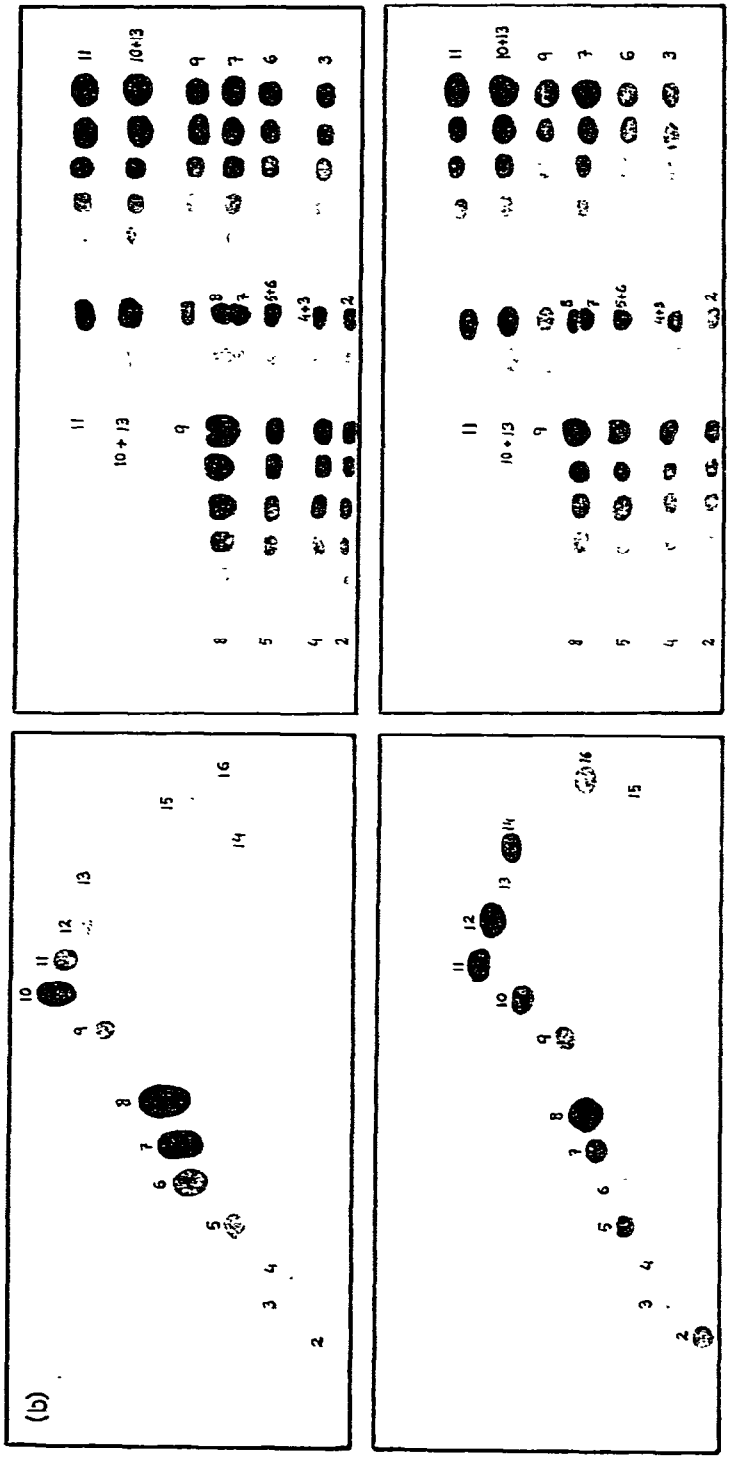


Fig. 3.

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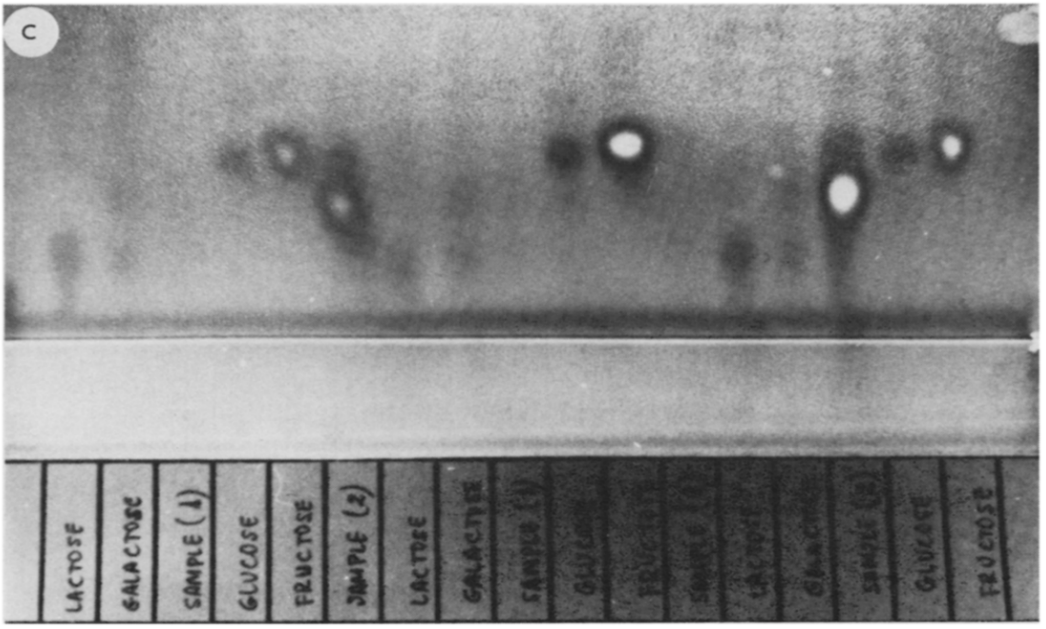

Fig. 3. Chromatograms. (a) The upper plates were developed at 7.5 cm with an evaporation time of 35 min. The lower plates were developed at 6 cm with an evaporation time of 15 min. (b) The identification number for each sugar is given in Table III. (c) The positions identified as sample 1 and sample 2 correspond respectively to a sample of a normal newborn infant and a galactosemic newborn infant in which, apart from galactose, there is also glucose as a result of parenteral feeding.

TABLE II

R_F VALUES MEASURED AS A FUNCTION OF EVAPORATION TIME ON PLATES ON WHICH INDIVIDUAL STANDARD SOLUTIONS OF SUGARS IN CONCENTRATIONS OF 500 mg PER 100 ml WERE CHROMATOGRAPHED

Development distance: 7.5 cm (measured from the separation line of the concentration zone).

Sugar	*Evaporation time (min)*						
	5	*10*	*15*	*20*	*25*	*35*	*49*
Stachyose	—	—	—	—	—	—	—
Raffinose	—	0.04	0.06	—	—	0.10	0.11
Lactose	0.04	0.09	0.10	0.07	0.09	0.20	0.11
Lactulose	0.06	0.11	0.11	0.09	0.09	0.21	0.27
Sucrose	0.13	0.17	0.19	0.10	0.19	0.38	0.43
Galactose	0.13	0.17	0.20	0.18	0.19	0.51	0.52
Glucose	0.19	0.27	0.29	0.27	0.28	0.55	0.56
Fructose	0.23	0.31	0.33	0.31	0.33	0.59	0.61
Arabinose	0.27	0.37	0.37	0.35	0.39	0.77	0.77
Xylose	0.44	0.51	0.50	0.51	0.57	0.92	0.94
3-O-Methylglucose	0.63	0.59	0.57	0.57	0.77	0.91	0.94
Xylulose	0.61	0.62	0.61	0.61	0.78	0.83	0.82
Ribose	0.49	0.54	0.54	0.57	0.64	0.77	0.85
Ribulose	0.54	0.27	0.58	0.59	0.72	0.31	0.39
Maltose	0.08	0.24	0.17	0.18	0.17	0.67	0.61
Mannose	0.23	0.27	0.38	0.36	0.41	0.37	0.40

The influence of the solvent strength was examined by varying the water content. The solvent strength chosen was that which provided the most suitable mobility for the sugars tested, and was less than that used previously[1,4].

The reason for using different evaporation times was to ensure distribution of the sugars so as to occupy the whole of the development zone of the plate. The best results were obtained with developments for 6 and 7.5 cm with evaporation times of 15 and 35 min, respectively. Greater distances gave poor results and longer evaporation times were needed. It was found that with continuous development at 6 cm the total time taken for the separation and assessment of the sugars was 1 h, as shown in Fig. 3. In Table III the detection limits and identification numbers of each sugar are given.

TABLE III

DETECTION LIMITS AND IDENTIFICATION NUMBERS OF THE SUGAR SAMPLES STUDIED

Identification No.	*Sugar*	*Detection limit (g/100 ml)**
1	Stachyose	–
2	Raffinose	0.05
3	Lactose	0.05
4	Lactulose	0.05
5	Sucrose	0.05
6	Galactose	0.10
7	Glucose	0.05
8	Fructose	0.05
9	Arabinose	0.05
10	Xylose	0.05
11	3-O-Methylglucose	0.05
12	Xylulose	0.05
13	Ribose	0.05
14	Ribulose	0.05
15	Maltose	0.05
16	Mannose	0.05

* The minimum concentration of each sugar tested was 0.05 g/100 ml. For some sugars the detection limit can be lower.

Ghebregzabher *et al.*[19] systematically studied the addition of boric acid[2,19] and phenylboronic acid to the mobile phase in the TLC of sugars of bioclinical interest; the acids were added to different mobile phases and solutions of standard sugars in aqueous isopropanol were applied. We, however, used real samples, which have completely different chromatographic characteristics. Our method provides information on the sugars that may be present in about the same period of time as that required for chromatographic separation (which is about equal to the minimum time required in ref. 19).

As can be seen from Table I galactose, glucose and fructose could be perfectly separated at a development distance of 6 cm and with an evaporation time of 5 min. Other sugars of clinical interest were separated without difficulty using a development distance of 6 cm and an evaporation time of 15 min (Fig. 3).

The R_F values did not follow a coherent pattern when the evaporation time was increased. We attribute this to the absence of control over the rate of evaporation, as the ambient temperature and air currents at the time were not stable.

CONCLUSIONS

The proposed continuous-flow TLC system allows the identification and assessment of sugars in urine impregnated on paper. This requires no previous treatment and, with elution and direct deposition of the paper on the plate, the whole analysis takes slightly over 1 h.

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REFERENCES

1 J. R. Alonso-Fernández, R. Quintas, J. Peña and J. M. Fraga, *International Symposium on Inborn Errors of Metabolism in Humans, September 2–5, 1980, Interlaken, Switzerland*, in press.
2 C. Auray-Blais, R. Giguere, P. Draper, D. Shapcott and B. Lemieux, *Clin. Biochem.*, 11 (1978) 235.
3 H. A. Harper, *Review of Physiological Chemistry*, Lange Medical Publications, Los Altos, CA, 12th ed., 1969, p. 11.
4 I. S. Menzies and J. N. Mount, *Med. Lab. Technol.*, 32 (1975) 269.
5 K. M. Haldorsen, *J. Chromatogr.*, 150 (1978) 485.
6 C. Szustkiewicz and J. Demetriou, *Clin. Chim. Acta*, 32 (1971) 355.
7 Y. Piemont, O. Michotey, J. Gindein and F. Lienhart, *Feuill. Biol.*, 18 (1977) 63.
8 I. S. Menzies and J. W. Seakins, in I. Smith and J. W. Seakins (Editors), *Chromatographic and Electrophoretic Techniques*, Heineman, London, 4th ed., 1976, p. 183.
9 J. A. Perry, *J. Chromatogr.*, 165 (1979) 117.
10 M. Brenner and A. Niederwieser, *Experientia*, 17 (1961) 237.
11 R. D. Bennett and E. Heftmann, *J. Chromatogr.*, 12 (1963) 245.
12 E. V. Truter, *J. Chromatogr.*, 14 (1964) 57.
13 T. M. Less, M. J. Lynch and F. R. Mosher, *J. Chromatogr.*, 18 (1965) 595.
14 G. J. Krol, G. R. Boyden, R. H. Moody, J. C. Comeau and B. T. Kho, *J. Chromatogr.*, 61 (1971) 187.
15 S. Hara and K. Mibe, *J. Chromatogr.*, 66 (1972) 75.
16 D. H. van den Eijnden, *Anal. Biochem.*, 57 (1974) 321.
17 S. Gocan, *Stud. Univ. Babes-Bolyai, Ser. Chem.*, 1 (1977) 12.
18 K. Y. Lee, C. F. Poole and A. Zlatkis, *Anal. Chem.*, 52 (1980) 837.
19 M. Ghebregzabher, S. Rufini, G. M. Sapia and M. Lato, *J. Chromatogr.*, 180 (1979) 1.