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# ANALYSIS OF CARBOHYDRATES IN BIOLOGICAL FLUIDS BY MEANS OF THIN LAYER CHROMATOGRAPHY

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SUMMARY

A new method for the monodimensional separation of sugars commonly found in biological fluids is described. Its simplicity, rapidity, reproducibility and low cost make it a technique suitable for routine screening and research in a clinical laboratory.

Application of the method to semi-quantitative analysis has also been evaluated and discussed. The results were surprisingly accurate when compared with spectrophotometric determinations.

## INTRODUCTION

Paper chromatography has been, until now, the method most often used for the qualitative analysis of sugars. The trouble with this method, however, is that it is too time-consuming. Although thin-layer chromatography is much more rapid, it has not been widely applied in this field because solvent systems that permit good sugar separations by the monodimensional technique have not yet been found.

In a preceding paper[1], we demonstrated the limits of monodimensional TLC and described a bidimensional technique that we had developed and which permitted good separations of the most complex sugar mixtures.

In clinical routine work, however, the bidimensional method is cumbersome. Moreover, sugar mixtures in biological fluids are less complex than those we proposed for analysis with the bidimensional technique. With these factors in mind, we saw the possibility of developing a monodimensional method whose efficiency would approximate that of the bidimensional technique when applied to sugar mixtures found in body fluids (which contain 5–6 sugars at most).

The present paper deals exclusively with the chromatography of sugars found in urine. With appropriate modifications in the sugar extraction technique, the same method can be applied to sugars found in other body fluids.

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## EXPERIMENTAL

*Preparation of the plates*

30 g of silica gel, TLC grade, with binder (Fluka G), is mixed with a 0.03 *M* $H_3BO_3$ solution (2.1 g of $H_3BO_3$ in 1 l of distilled water). The suspension obtained is applied in the usual manner to 20 × 35 cm glass plates (0.35 mm layer). These plates are dried for 24 h at room temperature and activated for 1 h at 110°.

*Preparation of the standard solutions*

Although initial experiments were carried out with two standard mixtures composed respectively of 11 and 7 sugars (as shown in Fig. 1), the mixtures which follow were simplified for the limited purposes of the present work. Semi-quantitative evaluation requires the repetition of the standard solution on the same plate at varying concentrations:

(1) *Nine-sugar solution A*. 15 mg each of raffinose, lactose, maltose, sucrose, galactose, levulose, ribose, xylose, rhamnose dissolved in 2 ml deionized water.

(2) *Nine-sugar solution B*. 10 mg each of the above sugars dissolved in 2 ml deionized water.

(3) *Nine-sugar solution C*. 10 mg each of the above sugars dissolved in 4 ml deionized water.

(4) *Glucose solution A*. 15 mg of glucose dissolved in 2 ml deionized water.

(5) *Glucose solution B*. 10 mg of glucose dissolved in 2 ml deionized water.

(6) *Glucose solution C*. 10 mg of glucose dissolved in 4 ml deionized water.

The investigator is free to change the composition of the mixtures for whatever specific project he may have on hand.

*Preparation of the samples*

Desalting is not necessary for normal urine, which can be applied directly to the plate [2,3]. Concentrated urine must be desalted. Desalting is accomplished by slowly passing the desired volume of filtered urine through a small column packed with an equal volume of AG 501 X8 (D) mixed bed resin (Bio-Rad Laboratories). The flow-rate should be about 20 drops per minute. When exhausted the resin changes color (from blue to gold), thus providing a visual indication of complete desalting. The column is then washed with a 5 % solution of acetic acid. The washing is continued, with at least one more volume of acetic acid solution, after the resin has completely turned to gold. The total eluate is taken to dryness under vacuum at 40°–50° in a rotary evaporator and is then diluted to 1 ml with deionized water.

*Application of the samples*

One microliter of each standard nine-sugar solution is applied with a micro-pipette to the chromatoplate. Spots are placed about 6 cm from each other and aligned about 2.5 cm above the bottom edge of the plate. The three glucose solutions of corresponding concentrations are then placed about 1.5 cm to the right of each spot. One microliter of the sample is then placed 2 cm to the right of any two of the pairs of standards. One microliter of the sample (at one-half the concentration of the other samples) is placed 2 cm to the right of the third pair of standards; the chromatoplate is then ready for development.

If a purely qualitative analysis is desired, only one pair of standards is needed.

*Development of the chromatogram*

The prepared plate is immersed in the usual manner in a tank containing the following developing solvent mixture: *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30). The front is allowed to run to the upper edge of the plate (total run: 32 cm). Time required is 6–7 h; room temperature is 20°.

*Detection of the spots*

The developed plate is dried at room temperature for a short time. It is then heated in an oven at 110° until the acetic acid odor can no longer be detected. The heated chromatogram is sprayed with a freshly prepared solution of 20 mg of naphthoresorcinol, 10 ml of ethanol and 0.2 ml of conc. $H_2SO_4$. The plate is then reheated for 5–10 min at 110°. The sugars appear as brightly colored spots (see Table I).

TABLE I

| *Carbohydrate* | *Approx. $R_F$ values × 100* | *Colors developed with naphthoresorcinol. reagent* |
|---|---|---|
| Raffinose | 19 | Orange-red |
| Lactose | 23 | Blue-violet |
| Maltose | 32 | Gray-violet |
| Sucrose | 36 | Crimson |
| Galactose | 40 | Gray |
| Levulose | 43 | Dark red |
| Ribose | 47 | Blue-green |
| Fucose | 50 | Purple-red |
| Xylose | 54 | Blue-green |
| Rhamnose | 60 | Cherry-red |
| Glyceraldehyde | 71 | Gray |
| Melibiose | 24 | Violet |
| Turanose | 33 | Purple-red |
| Mannoheptulose | 43 | Violet |
| Glucose | 46 | Gray-violet |
| Lyxose | 53 | Blue-green |
| Mannose | 60 | Violet |
| Dihydroxyacetone | 64 | Gray |
| Sedoheptulose | 38 | Violet |
| Erythrose | 53 | Green |
| Sorbose | 46 | Red |
| Trehalose | 31 | Blue |
| Arabinose | 45 | Blue-green |

## RESULTS AND DISCUSSION

In our efforts to develop a monodimensional method that approximates the efficiency of the bidimensional technique, we tried out a wide variety of the solvent systems reported in the literature, but were unable to obtain even remotely satisfying results.

The best results were obtained with the solvent described in the experimental section which, under the best conditions, separated the following six sugars on a standard 20 × 20 cm plate: lactose, maltose, galactose, levulose, ribose, rhamnose.

Fig. 1. Monodimensional chromatography of standard mixtures of carbohydrates on a 20 × 35 cm plate. Solvent system: *n*-butanol–ethyl acetate–isopropanol–acetic acid–water (35:100:60:35:30). 1, 3 and 5 = 8, 4 and 2 $\mu$g per spot respectively of (from bottom to top): raffinose, lactose, maltose, sucrose, galactose, levulose, ribose, fucose, xylose, rhamnose, glyceraldehyde. 2, 4 and 6 = 8, 4 and 2 $\mu$g per spot respectively of (from bottom to top): melibiose, turanose, mannoheptulose, glucose lyxose, mannose, dihydroxyacetone.

Some of these spots, however, were so close together that when the amount of sugar in each spot exceeded 4 $\mu$g, separation was effectively impaired. We therefore lengthened the run by using 20 × 35 cm plates and were able to obtain up to 11 distinctly separated spots: raffinose, lactose, maltose, sucrose, galactose, levulose, ribose, fucose xylose, rhamnose and glyceraldehyde. Other sugars were not clearly separated if added to the mixture, and separation of the remainder of the 23 sugars shown in Table I was achieved only by dividing them into three other solutions: (1) melibiose–turanose–mannoheptulose–glucose–lyxose–mannose–dihydroxyacetone; (2) sedoheptulose–erythrose–sorbose–trehalose; (3) arabinose.

From a clinical point of view, the 11-sugar standard mixture is incomplete, since glucose, one of the biologically most important sugars, is lacking. This sugar overlaps the levulose and ribose spots, so routine clinical analysis requires the addition of at least a separate glucose standard to the 11-sugar standard mixture.

When rapid screening is desired, the 20 × 20 cm plate can be used, if it is kept in mind that all dubious cases must be repeated with the longer plate.

Preparation of the samples varies according to the amount of salt present in the urine. Normal urine may be spotted directly onto the plate, since the ion concentration is too low to alter appreciably the migration of the sugars[3]. Direct spotting of normal urine, however, shows only those sugars present in relatively large quantities, and is therefore only suitable for such routine work as testing for massive glycosurias. If sugars present in small quantities are to be analysed, it is necessary to concentrate the urine, which brings the saline content up to levels which interfere with the migration of the sugars: the $R_F$ values are lowered and the spots tail. In this case, it is therefore necessary to desalt the sample.

The desalting method described in the experimental section gives the best assurance of quantitative recovery of the sugars.

The passage of one volume of filtered urine through an equal volume of resin —without an acetic acid wash — resulted in complete desalting, but the sugar content of the eluate was lower than in the original urine. All the sugar concentrations were decreased by varying percentages (between 30% and 50%) and some of them disappeared altogether. Extensive washing of the used resin with water did not help[4].

We tested the amount of sugar retention by passing 10 ml of urine through a small column packed with 10 ml of mixed bed resin. The column was then washed with 20 ml deionized water. The desalted urine plus the washings were then taken to dryness under vacuum with a rotary evaporator and diluted to 1 ml with deionized water (Sample I). At this point, the resin was washed with 20 ml of a 5 % acetic acid solution, taken to dryness and diluted, as above, to 1 ml (Sample II).

One microliter each of Samples I and II were spotted on a plate next to 1 $\mu$l of non-desalted urine concentrated 10:1 (Sample III). After development, we observed that Sample I contained a lower percentage (about 50 %) of sugars than Sample III. Sample II contained the difference between Sample III and Sample I.

A parallel experiment was carried out with a $CO_2$-saturated resin[4], and, again, Sample I contained a lower percentage of sugars than Sample III, but in this case, although Sample I contained a higher percentage of sugars than in the preceding experiment, the resin still retained between 15 %–25 % of the sugars.

A variation of the above-mentioned two experiments, with the resin added directly to the urine[4–7], yielded an even lower percentage of sugars in Sample I.

To sum up:

(1) Acetic acid washing is necessary in order to obtain complete recovery of the sugars from the resin.

(2) $CO_2$ saturation of the resin before desalting is not only ineffective, but, by changing the color of the resin indicator, prevents visual control of the progressive exhaustion of the resin during desalting.

Concentration of urines and eluates must be done under vacuum and at temperatures not higher than 40–50°. The easiest and most rapid way to achieve evaporation is with a rotary evaporator and the specially shaped flask in Fig. 2.

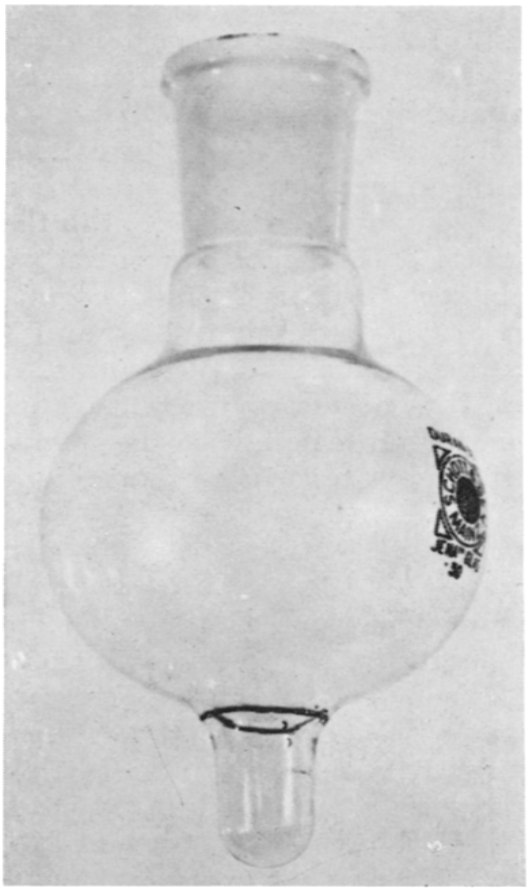

Fig. 2. Specially shaped flask for the rotary evaporator. The reservoir is marked to 1 ml.

The chromatography of sugars as described in the experimental section is applicable not only to qualitative analysis, but also to quantitative evaluation. The sensitivity of the method is such that spots of less than 1 $\mu g$ can be detected. In photographs of the plates, these small quantities are not visible, but on the plates themselves the spots can easily be seen. Moreover, the intensity of color and the size of each spot is proportional to the quantity of sugar present.

It should be noted that the spots with higher $R_F$ values are more subject to diffusion (Fig. 1) and for this reason, plus the fact that the color of each sugar is quite different from the next, it is necessary to prepare a standard chromatogram where each sugar is shown at different levels of dilution.

Comparison of a developed chromatogram showing regularly increasing con-

centrations of the standard sugar mixture (1 $\mu$g through 7.5 $\mu$g of each sugar) with the plate developed as in the experimental section, makes quantitative visual appraisal of the sugars in the sample much more accurate. Either a color photograph or a chromatogram preserved with Neatan* can be used as a standard for comparison.

The maximum limit of visual quantitative evaluation is about 8 $\mu$g per spot. If, in a sample, one or more spots contains more than 8 $\mu$g, it is advisable to prepare another plate with a series of increasing dilutions of the sample in question.

We controlled the accuracy of the evaluation of the chromatogram by comparing the visually deduced values of individual sugars with the spectrophotometric determination of the same**. In general, visual evaluation was 75 % to 95 % accurate.

The margin of error can be further reduced by following the procedure described in the experimental section: a given concentration of the sample is spotted next to two of the standard solution pairs, while the same sample, at one-half the given concentration, is spotted next to the third pair of standard solutions. Triple evaluation of the spots insures elimination of experimental error.

## REFERENCES

1 M. LATO, B. BRUNELLI, G. CIUFFINI AND T. MEZZETTI, *J. Chromatog.*, 34 (1968) 26.
2 E. RAGAZZI AND G. VERONESE, *Farmaco (Pavia), Ed. Prat.*, 18 (3) (1962) 152.
3 E. GUILLOUX AND S. BEAUGIRAUD, *Bull. Soc. Chim. France*, (1965) 259.
4 L. WOOLF, *Nature*, 171 (1953) 841.
5 G. MASERA AND H. KASER, *Minerva Pediat.*, 16 (1964) 14.
6 H. KASER AND G. MASERA, *Schweiz. Med. Wochschr.*, 94 (1964) 158.
7 H. BICKEL, *J. Pediat.*, 59 (1961) 641.

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