ION EXCHANGE CHROMATOGRAPHY OF POLYOLS

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Polyols have been detected in many biological fluids and tissues[1]. A systematic study of polyols has been hindered by lack of suitable methods of separation, although chromatographic methods have been devised for particular problems such as the determination of glycerol[2] and propylene glycol[3] in tobacco.

The most widely used method is based on the work of KHYM AND ZILL[4] who separated polyols as their borate complexes on strongly basic anion exchange resins in the borate form. The disadvantage of this procedure lies in the use of dilute borate buffers for elution with the consequent production of broad solute peaks.

Analysis of polyols is also hampered by lack of a specific analytical method; assays based on the measurement of periodate uptake are relatively insensitive and sugars interfere to a large extent.

In the present work these disadvantages have been overcome by elution from ion exchange resins with concentrated borate buffers and by estimating the formaldehyde produced by periodate oxidation of polyols.

These techniques have been applied to the analysis of urinary polyols.

MATERIALS AND METHODS

Chemicals

The author is indebted to Dr. C. E. STICKINGS of Imperial College, London, for a sample of L-threitol.

Glycerol was used without further purification. Other commercially available polyols were purified by recrystallisation from absolute or aqueous ethanol. After repeated recrystallisations some polyols were found to be impure when analysed by the column procedure described here. For recovery experiments it was found convenient to prepare these polyols in solution by reducing the corresponding Analar grade aldose sugar with sodium borohydride by the method of ABDEL-AKHER, HAMILTON AND SMITH[5]. The disappearance of reducing sugar was followed to completion by measuring reducing power by the method of PARK AND JOHNSON[6]. Suitably diluted samples of these solutions were used without further treatment.

Analar grade sodium metaperiodate and chromotropic acid "for formaldehyde analysis" were used. Bisulphite solutions were prepared from B.D.H. ampoules. $^{14}C_1$-Mannitol was obtained from the Radiochemical Centre, Amersham.

Column chromatographic procedure

De-Acidite FF (3–5 cross-linked, passing 200 mesh: Permutit Co. Ltd.) was

exhaustively washed with water and the fines removed by decantation. The resin was regenerated in large glass columns by passing 5 column volumes of 2*N* sodium hydroxide. After washing excess alkali from the column with water, the resin was converted into the borate form by passing 5 column volumes of 0.5*M* potassium tetraborate. Excess borate was washed from the resin with distilled water; the resin was then extruded and stored moist.

The analytical columns were jacketed and were 60 cm in length and 0.8 cm internal diameter. The resin was thoroughly degassed at 40–50° on a vacuum pump and then poured as a thick slurry (0.5 vol. water) to give a column 50–55 cm in length. Water at 35° circulated through the column jacket as the resin was poured and throughout the chromatographic procedure. The sample, dissolved in 1–2 ml of 0.1*M* borate pH 9–10, was applied when excess liquid had just drained to the top of the resin bed. In the same way 1–2 ml washings were applied. Eluting buffer was then immediately pumped onto the column *via* a small de-aeration tube[7] placed in a circulating water bath at 35°. The pump was adjusted to give a column flow rate of 25 ml per h and 5 ml fractions were collected using an Isco volumeter (Shandon Scientific Supplies). Eluting buffers were 0.18 and 0.36*M* boric acid adjusted to pH 9 with triethylamine. The column was eluted with 0.18*M* buffer up to fraction 80 when 0.36*M* buffer was introduced. Conductivity measurements showed that the 0.36*M* buffer emerged from the column at fraction 89–90.

Analysis of column eluates

Following periodate oxidation, formaldehyde was estimated by a modification of the methods described by WEST AND RAPAPORT[8] and by BARTLETT[9].

The reactions were carried out in Pyrex test tubes. To 2 ml samples were added 0.5 ml 0.01*M* sodium metaperiodate in *N* sulphuric acid. After mixing, the solutions were allowed to stand for at least 10 min at room temperature. Next 0.2 ml 10% (w/v) sodium bisulphite was added with immediate mixing followed by 0.2 ml 2% (w/v) aqueous chromotropic acid solution. Finally 3 ml conc. sulphuric acid was added from a burette; the solutions were mixed well using a vortex mixer and the tubes were then placed in a boiling water bath for 1 h. After some 10 min in the bath the tubes were briefly removed and the contents again well mixed with the vortex mixer before the tubes were returned to the bath: incomplete mixing at this stage resulted in the formation of a precipitate which interfered in the spectrophotometer readings. The timing for both the periodate oxidation and the colour development stage in the water bath was not found to be critical; 10–30 min sufficed for the oxidation and 45–60 min for the colour development. Reagents were freshly prepared and added by automatic pipette or syringe.

In the presence of borate, full development of the red colour was inhibited by an amount proportional to the amount of borate present. For a constant level of borate however, the colour intensity was found to be directly proportional to the amount of polyol oxidised. The polyols examined in the present procedure were eluted by 0.18*M* or 0.36*M* borate solutions; it was found convenient to dilute 1 ml samples of column fractions to 2 ml with either 0.18*M* buffer or water so that all samples were 0.18*M* with respect to borate before addition of periodate. The resulting colours were then related to a standard curve prepared using pure erythritol dissolved in 0.18*M* buffer. Colours were read at 570 mμ in a Unicam SP 500 spectrophotometer;

in the reaction mixture described 0.1 μmole polyol (yielding 2 moles formaldehyde per mole) gives an extinction of 0.5 with a path length of 1 cm.

Of the common sugars which were tested in this assay only fructose interfered to any extent giving an extinction of 0.1 per 0.1 μmole.

Radioactivity measurements

Radioactivity was counted in a Packard Tri Carb scintillation counter. 0.2 ml samples of column fractions were diluted with 2.8 ml absolute alcohol and 7 ml of scintillation fluid. The scintillation fluid contained 5 g 2,5-diphenyloxazole and 0.3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l of toluene.

Paper chromatography

Those fractions which on analysis were found to contain a solute peak were combined. Triethylamine was removed with Zeo-Carb 225 (Permutit Co. Ltd.) and the boric acid was distilled off as methyl borate by repeatedly evaporating to dryness in the presence of methanol[4]. The resulting residues were dissolved in 0.1 ml water and spotted on to sheets of Whatman No. 3 MM paper. The papers were irrigated for 18 h by descending flow with the solvent, ethyl acetate–pyridine–water saturated with boric acid (12:5:4 by vol)[10]. Chromatograms were allowed to dry in air; polyols (and sugars) were revealed using the anisidine–periodate dip method of BEAN AND PORTER[11].

Preparation of urine samples for analysis

Urines were collected over a 24 h period and kept at 4° with a few drops of toluene added as preservative. Samples were processed within 24 h of the last collection. 10 ml portions were added to 15 ml of moist Bio-Deminrolit which had been converted to the HCO_3^- form by passing an excess of CO_2 through a suspension of the resin in water. The urine–resin slurry was magnetically stirred for 30 min after which the resin was filtered off and washed well. The filtrate and washings were freeze-dried and kept in a deep freeze. For analysis the residues were dissolved in borate buffer and applied to the column as described.

RESULTS

Under the conditions described a mixture containing nine polyols is almost completely resolved into nine discrete solute peaks (Fig. 1a). The effect of temperature on the separation was particularly marked; at 25° the affinity of the polyol–borate complexes for the resin is lowered so that many of the polyol peaks overlap, whilst at 45° polyols are so strongly bound to the resin that the time required for elution is impractical. At 35° only mannitol and galactitol overlap; when the less commonly occurring polyols, rhamnitol and fucitol are included in the mixture they overlap arabitol and glucitol respectively (Table I).

Recoveries of individual polyols after the separation of various mixtures are shown in Table I, which also indicates the fraction numbers at the peak positions. These positions varied by up to ten fractions from the values quoted but the order of elution of the polyols remained constant.

Absorption of hexitols by strongly basic ion exchange resins was reported by ROSEMAN *et al.*[12]. Since the procedure for desalting urine described earlier involved the use of a strongly basic resin, the effect of this procedure on the recoveries of

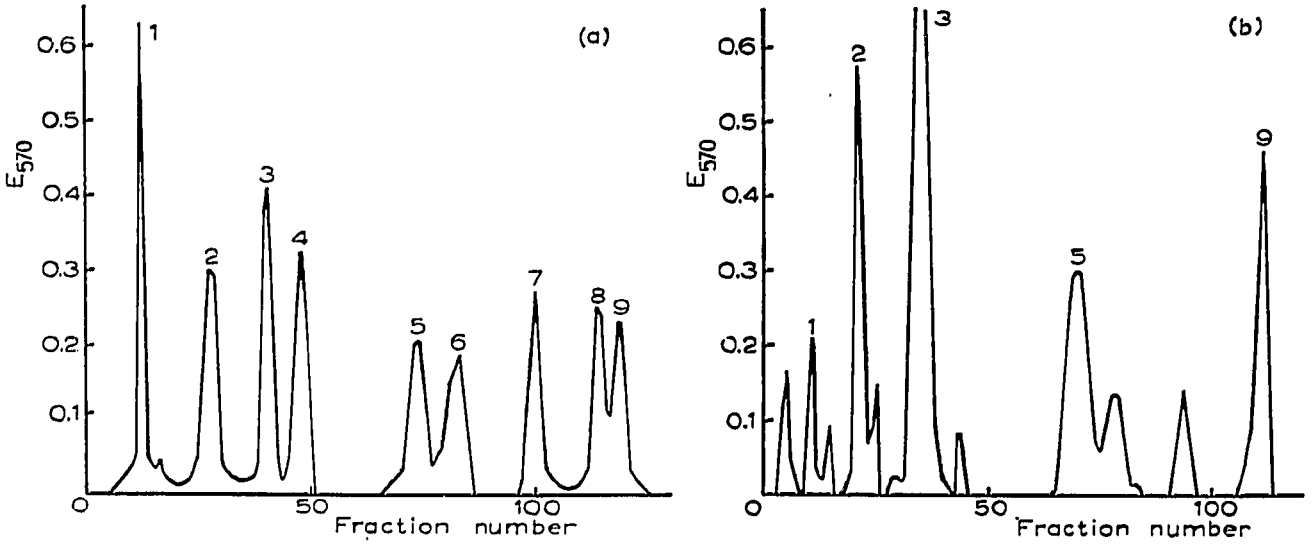

Fig. 1. Elution curves of polyols in a synthetic mixture (a), and from 10 ml of a normal urine (b); conditions as described in the text. Peaks are identified as follows: 1 = glycerol; 2 = threitol; 3 = erythritol; 4 = xylitol; 5 = arabitol; 6 = ribitol; 7 = glucitol; 8 = galactitol; 9 = mannitol.

micromole amounts of polyols was therefore examined. The results presented in Table II indicate that appreciable losses of all the common polyols resulted from the use of the desalting procedure.

The method has been applied to the analysis of urines from apparently healthy individuals: a typical elution pattern is shown in Fig. 1b. Five polyols were consistently found in the urines which were examined; glycerol, threitol, erythritol, arabitol, and mannitol. With the exception of glycerol the polyols were identified from their elution positions and by paper chromatography; mannitol was also identified by co-chromatography with $^{14}C_1$-mannitol. The method described earlier[4] for removing boric acid from column fractions resulted in the complete loss of glycerol which was therefore identified solely by its elution position.

The 24 h excretion values of the five polyols which were found are presented in Table III.

TABLE I

RECOVERIES AND PEAK FRACTION POSITIONS OF POLYOLS

Recoveries are expressed as the average percentage recovery ± standard deviation. The number of determinations is given in brackets. The peak fraction number is the average of at least 5 determinations.

Polyol	*Recovery*	*Peak fraction number*
Glycerol	108 ± 5.1 (6)	12
Threitol	100 ± 3.9 (7)	26
Erythritol	92.2 ± 4.7 (7)	39
Xylitol	94.6 ± 11.2 (8)	46
Arabitol	89.2 ± 4.2 (8)	71
Ribitol	84.1 ± 5.9 (10)	79
Glucitol	89.5 ± 14.1 (13)	101
Galactitol	92.0 ± 7.3 (5)	114
Mannitol	99.0 ± 14.5 (5)	117
Fucitol	95.6 ± 7.0 (6)	99
Rhamnitol	89.5 ± 4.8 (6)	67

TABLE II

RECOVERIES OF POLYOLS AFTER DESALTING BY THE PROCEDURE DESCRIBED IN THE TEXT

Recoveries are presented as percentages of untreated samples. The values for three separate experiments are shown with the mean value in brackets.

Polyol	*Recovery*
Glycerol	70, 68, 60 (66)
Threitol	62, 60, 55 (59)
Erythritol	60, 49, 53 (54)
Xylitol	53, 64, 60 (59)
Arabitol	72, 60, 68 (67)
Ribitol	70, 77, 73 (73)
Glucitol	38, 48, 50 (45)
Galactitol	45, 43, 36 (41)
Mannitol	44, 36, 40 (40)

TABLE III

EXCRETION OF GLYCEROL, THREITOL, ERYTHRITOL, ARABITOL AND MANNITOL IN μMOLES PER 24 HOURS

The figures for each polyol were calculated from elution curves obtained by the methods described in the text and were corrected for losses incurred in the desalting procedure.

Sample	*Sex*	*Age*	*Glycerol*	*Threitol*	*Erythritol*	*Arabitol*	*Mannitol*
1	F	19	33	164	724	252	307
2	F	17	108	495	715	243	540
3	F	20	94	156	690	315	285
4	F	20	45	122	600	313	1390
5	M	30	55	250	990	400	159
6	M	23	68	198	1060	323	120
7	M	16	124	156	695	425	385
8	M	33	73	203	610	485	190
9	M	37	42	78	445	122	122

DISCUSSION

Recently gas chromatographic methods have been described (see for example SWEELEY *et al.*[13]), for the analysis of sugars and polyols which offer distinct advantages in terms of speed and sensitivity over the methods described here. However, ion exchange methods are useful in the analysis of unknown mixtures as the analytical procedures used often serve to partly characterise solute peaks which can also be collected for identification by other methods. WELLS *et al.*[14] described a method for the analysis of sugars and polyols in serum and urine by gas chromatography of the corresponding trimethylsilyl ethers; mannitol was detected as a constituent of normal urine.

The presence of mannitol, erythritol[15], arabitol[16] and threitol in normal urine may be significant since MCCORKINDALE AND EDSON[17] found that rat liver polyol dehydrogenase did not oxidise mannitol, erythritol or arabitol; the human urinary polyols may represent minor end products of carbohydrate metabolism.

The occurrence of threitol is noteworthy as this polyol has previously only been detected in bacterial[18], and plant material[19], although BATT *et al.*[20] showed that in rats ^{14}C-L-erythrulose is converted in part to threitol. The guinea pig liver enzyme reported by HOLLMAN AND TOUSTER[21], which converts D-xylulose to xylitol will also convert L-threitol to L-erythrulose. In humans threitol metabolism may possibly be linked to that of L-erythrulose but little is known about the metabolism of this sugar.

The fact that only small amounts of polyols are normally found in urine suggests that rapid screening methods for urinary polyols may be valuable in clinical diagnosis since it is known that large amounts of arabitol are excreted by pentosuric individuals[16]. Similarly galactitol has been isolated in quantity from the urines of patients with galactosemia[22] and from the urine of an individual with a possible generalised deficiency of the enzyme galactokinase[23].

SUMMARY

A method is described for the separation of 0.5–1.0 micromole amounts of some common polyols by ion exchange chromatography. Application of the method to the analysis of urinary polyols has confirmed the presence of arabitol, erythritol and mannitol in normal human urine; threitol has also been detected. The 24 h excretion values of some polyols are presented.

REFERENCES

1 O. TOUSTER AND D. R. D. SHAW, *Physiol. Rev.*, 42 (1962) 181.
2 S. J. PATTERSON, *Analyst*, 88 (1963) 387.
3 R. L. CLEMENTS AND S. J. PATTERSON, *Analyst*, 89 (1964) 17.
4 J. X. KHYM AND L. P. ZILL, *J. Am. Chem. Soc.*, 74 (1952) 2090.
5 M. ABDEL-AKHER, J. K. HAMILTON AND F. SMITH, *J. Am. Chem. Soc.*, 73 (1951) 4691.
6 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149.
7 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
8 C. D. WEST AND S. RAPAPORT, *Proc. Soc. Exptl. Biol. Med.*, 70 (1949) 141.
9 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 459.
10 C. GRADO AND C. E. BALLOU, *J. Biol. Chem.*, 236 (1961) 54.
11 R. C. BEAN AND G. G. PORTER, *Anal. Chem.*, 31 (1959) 1929.
12 S. ROSEMAN, R. H. ABELES AND A. DORFMAN, *Arch. Biochem. Biophys.*, 36 (1952) 232.
13 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.
14 W. W. WELLS, T. CHIN AND B. WEBER, *Clin. Chim. Acta*, 10 (1964) 352.
15 O. TOUSTER, S. O. HECHT AND W. M. TODD, *J. Biol. Chem.*, 235 (1960) 951.
16 O. TOUSTER AND S. O. HARWELL, *J. Biol. Chem.*, 230 (1958) 1031.
17 J. MCCORKINDALE AND N. L. EDSON, *Biochem. J.*, 57 (1954) 518.
18 C. L. HU, E. A. MCCOMB AND V. V. RENDIG, *Arch. Biochem. Biophys.*, 110 (1965) 350.
19 E. A. MCCOMB AND V. V. RENDIG, *Arch. Biochem. Biophys.*, 103 (1963) 84.
20 R. D. BATT, F. DICKENS AND D. H. WILLIAMSON, *Biochem. J.*, 77 (1960) 281.
21 S. HOLLMAN AND O. TOUSTER, *J. Biol. Chem.*, 225 (1957) 87.
22 W. W. WELLS, T. A. PITMAN AND T. J. EGAN, *J. Biol. Chem.*, 239 (1964) 3192.
23 R. GITZELMANN, H. C. CURTIUS AND M. MULLER, *Biochem. Biophys. Res. Commun.*, 22 (1966) 437.