Journal of Chromatography, 337 (1985) 29—36
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2354

GEL PERMEATION CHROMATOGRAPHY WITH INTERFERENCE REFRACTOMETRY FOR THE RAPID ASSAY OF POLYDISPERSE DEXTRANS IN BIOLOGICAL FLUIDS

MARION P. CULLEN*, CHARLES TURNER and GEORGE B. HAYCOCK

United Medical & Dental School of Guy's & St. Thomas' Hospitals, Department of Paediatrics, 12th floor, Guy's Tower, Guy's Hospital, St. Thomas Street, London SE1 9RT (U.K.)

(First received June 7th, 1984; revised manuscript received August 28th, 1984)

SUMMARY

A semi-automatic system incorporating an ultra-sensitive interference refractometer coupled to a dual-column gel permeation apparatus has been devised for measurement of the molecular size distribution of dextrans in small samples of serum and urine. The system was calibrated with seventeen defined dextran fractions with a range of 1200—250,000 weight average molecular weight ($\bar{M}_w$). Urine samples were prepared for analysis by passage through small ion-exchange columns; serum was pretreated by precipitation with trichloroacetic acid and centrifuged before the ion-exchange treatment. Internal standard (dextran, $2 \cdot 10^6$ $\bar{M}_w$) was added to each sample before pretreatment. Data were obtained in a form suitable for computerised analysis.

INTRODUCTION

The objective of this investigation was to develop a rapid, reliable, semi-automatic method for the separation and estimation of different molecular-weight fractions of neutral dextran in serum and urine. Several groups [1—3] have published methods for the estimation of the molecular size distribution of clinical dextran. The analytical techniques employed by these workers have several disadvantages. The turbidimetric assay of Arturson and Wallenius [1] is too expensive for general use. The conventional gel permeation chromatography employed by other groups [2—4] is slow in operation. Leaching out of dextran-based column packings may contaminate the sample [2]. The colorimetric reactions used for the determination of chromatographed dextran are relatively insensitive, involve handling of large volumes of boiling, concentrated mineral acids and are difficult to automate. A number of the clinical

0378-4347/85/$03.30

studies of membrane permeability have relied on gel permeation chromatography using a heterogeneous calibration mixture of proteins and polysaccharides.

Since the publication of these techniques, mechanically stable column packings have been developed allowing the application of high-speed, high-pressure gel permeation chromatography in aqueous media [5—8]. Estimation of dextran concentration in solutions using interference refractometry has been shown to be highly sensitive, reproducible and to produce results comparable with chemical assays [9]. The combination of these methods was considered likely to offer a significant improvement in speed, sensitivity and cost compared with other existing assays. The continuous measurement of column eluates using a flow-through interference refractometer also allows on-line data collection and reduction using microcomputer techniques. Calibration of gel permeation columns using defined dextran fractions derived from the same organism (*Leuconostoc mesenteroides*) as clinical dextran removes the uncertainty associated with heterogeneous calibration standards.

This communication describes the assembly of the semi-automatic system, its calibration with dextran standards and preparation of the biological samples for analysis using an interference refractometer.

EXPERIMENTAL

Apparatus and materials

Pre-packed columns containing hydrophilic polymer based, semi-rigid gels connected in series were used. TSK columns were used throughout. A pre-column (75 mm × 7.5 mm I.D., GWPH) was followed by two analytical columns (both 600 mm × 7.5 mm I.D., one 5000 PW and one 3000 PW); all columns were purchased from Toyo Soda Manufacturing (Tokyo, Japan). The two analytical columns were water-jacketed and maintained at 33°C. Two in-line 2 μm sieve size filters were incorporated in the stainless-steel lines (1.16 mm O.D. and 0.51 mm I.D.) to protect the columns from contamination; both disposable and re-usable frits were used (Jones Chromatography, Llanbradach, U.K. or Millipore, Harrow, U.K.). The eluent was 0.02% aqueous sodium azide (Fluka, Buchs, Switzerland). To ensure freedom from particulate material and to remove dissolved air it was made up in freshly distilled water and vacuum-filtered with all glass apparatus incorporating disposable filters (Millipore, HAWP 04700). Samples were injected into the system using a sample processor (Model 710B WISP Sample Processor, Waters Assoc., Milford, MA, U.S.A.); samples were prefiltered through 0.45-μm filters (Millex-HV) from Millipore and degassed by sonication for 5 min (Decon Ultrasonics System Model FS100, Hove, U.K.). The volumes of sample injected were programmed in accordance with the anticipated concentration of dextran in the solutions. An interference refractometer equipped with an 8-mm cell using the wavelength of 546 nm and set at × 50 range was used to assay the ratio of the concentrations of dextran in the samples relative to the known concentration in standard solutions (Optilab 902, Tecator AB, Hoganas, Sweden). The detector cell was maintained at 33°C with a water circulator (FH 15 Flow Heater, Grants Instruments Cambridge, Cambridge, U.K.). Two electronic integrators (LDC 308, Laboratory Data

Control, Riviera Beach, FL, U.S.A., or SP 4270, Spectra Physics, San Jose, CA, U.S.A.) were connected to the interference refractometer using 1 V output. Either integrator was set to receive the signal from the interference refractometer and they were programmed to compute the area under the peak and/or in a "slice" of the dextran curve or peak taken every 30 sec. The data from the SP 4270 integrator were transmitted via an RS-232-C interface to an Apple 2+ microcomputer for calculation of results. The analogue signal from the interference refractometer was fed in parallel to an LKB 2210 recorder (Bromma, Sweden) set at 2 mm/min chart speed. The system was pumped at a constant flow-rate (0.81—0.82 ml/min) by means of a Constametric III HPLC unit (Laboratory Data Control). A pulse damper and pressure gauge were connected to the output of the pump by a stainless-steel capillary T-joint; working pressures did not exceed 3.8 MPa (550 p.s.i.). The entire system was started and controlled by the programmable sample processor. Dextran 40 injection BP in 5% dextrose was used as the polydisperse dextran (Lomodex, Fisons Pharmaceutical, Loughborough, U.K.).

Calibration of the system

Direct calibration of the system was achieved by the use of a series of seventeen dextran fractions (Dr. R. Gibbs, Fisons Pharmaceutical, Crewe, Cheshire, U.K., and Dr. K. Granath, Pharmacia Chemicals, Uppsala, Sweden). Each fraction was prepared by weighing the dry powder and made up as a 10% (w/v) stock solution in 0.02% sodium azide; all stock solutions were held at 8°C. Working concentrations of 0.1% were prepared just prior to running on the columns. Each calibration fraction was subjected to analysis at least nine times, and at least three different volumes of sample were injected: 25, 50 and 75 μl. The calibration standards were loaded on to the columns in amounts ranging from 0.01 to 0.07 mg. The weight average molecular weight ($\overline{M}_w$) ranged from 1200 to 250,000; these values had been determined by low-angle laser beam analysis (Fisons). Using the data of Granath and Kvist [2] (Table I) Stokes radii were calculated for those dextran fractions having $\overline{M}_w$ values within the published range. A dextran polymer with an $\overline{M}_w$ in excess of 2,000,000 was used to determine the void volume (V_0), while glucose (the monomer of which dextran is a polymer) was used to define the total separation volume (V_t) of the series of columns incorporated in the system.

TABLE I

LITERATURE DATA USED TO CALCULATE STOKES RADII OF THE DEXTRAN CALIBRATION FRACTIONS [2]

$\overline{M}_w$	Stokes radius (nm)	$\overline{M}_w$	Stokes radius (nm)
10,000	2.33	58,000	5.30
13,200	2.65	76,000	6.05
19,300	3.18	96,000	6.75
27,800	3.77	130,000	7.80
36,000	4.25	147,000	8.20
48,300	4.85		

For each defined fraction the elution volume at which 50% of the material had emerged was designated the 50% volume (V_{50}). Using these values a distribution coefficient (K_{av}) was determined after the concept developed by Laurent and Killander [10].

$$K_{av} = \frac{V_{50} - V_0}{V_t - V_0}$$

The values obtained when the sized dextran fractions were run separately were used to calibrate the dual-column system; these calculations can also be made on the basis of retention time.

Preparation of serum samples

Serum samples were stored at −70°C. The effective removal of serum components which could contaminate the analytical columns or invalidate the assay of the content of dextran in the sample posed considerable difficulties. Precipitation with zinc sulphate failed to clear the serum of residual components as evidenced by the uninterpretable chromatograms. It was, however, found that serum could be effectively prepared for analysis by first precipitating it with 10% trichloroacetic acid (TCA). A solution of a large dextran fraction ($>10^6$ $\bar{M}_w$) as for V_0 was used as an internal standard to allow correction for dilution or losses during sample preparation and as a check on chromatographic conditions. Samples were treated as follows: 0.25 ml distilled water (or an aliquot of dextran infusate), 0.25 ml internal standard, 0.50 ml serum, and 3.50 ml of 10% TCA were well mixed after each addition and finally homogenised with a Vortex mixer; after standing for 10 min the suspension was centrifuged (840 *g*) for 5 min and the supernatant was decanted. Recovery volume was 4.0 ± 0.1 ml.

As the dual-column packing could be damaged by excessive acid, serum samples had to be neutralised prior to being run on the analytical system. Removal of TCA by extraction with diethyl ether was found to be unsatisfactory. A suitable means of neutralising the samples was found by filtering them through separate anion and cation resins. Analytical-grade anion-exchange resin, AG 2×8 (Cl^-) and cation-exchange resin AG 50W-X2 (H^+) both 100—200 mesh obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) were used. The resins were pretreated with 1 mol/l sodium hydroxide (anion) or 1 mol/l hydrochloric acid (cation); 2.0 ml of resin was then placed in a polypropylene column with a total volume of 11 ml (Econo-Column, Bio-Rad Labs.); resins were washed with freshly prepared boiled, glass-distilled water, and washing was continued until the eluent from the column was neutral when tested with Universalindikator pH 1—14 (E. Merck, Darmstadt, F.R.G.). Serum supernatant was poured on to the anion column and passed directly to the cation column from which it emerged at a neutral pH.

Preparation of urine samples

Urine samples were held at 8°C. Removal of urine components which interfered with the detection of the dextran was undertaken. Precipitation with zinc sulphate completely failed to yield a solution capable of being analysed by means of interference refractometery. Methanol precipitation, with or without

addition of sodium chloride to the urine, was also ineffective. Treatment using the same type of ion-exchange columns as for serum was found to yield satisfactory results. The urine test samples were prepared as follows: 0.25 ml internal standard and 4.25 ml urine.

The urine preparation was passed through the cation column before entering the anion column. All samples were then filtered through Millex filters, sonicated and loaded into the autosampler which was already programmed for analysis.

RESULTS

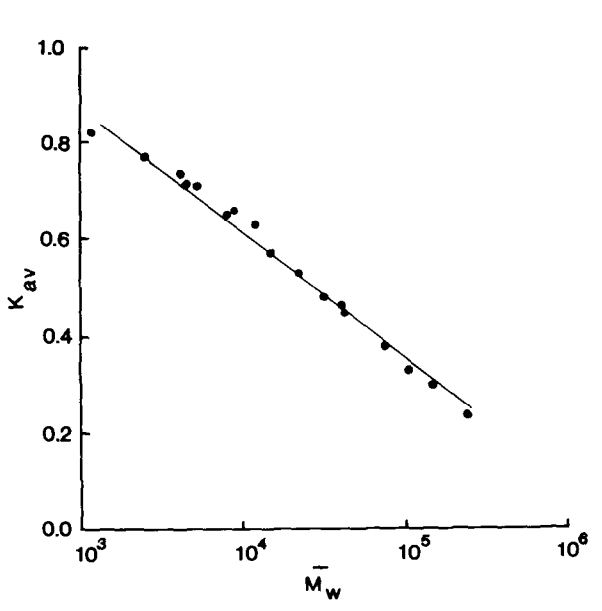
The elution patterns of the sized fractions of dextran used to calibrate the dual columns were Gaussian. Table II illustrates the V_{50} values found for the elution of the standards and the K_{av} values calculated therefrom. A plot of K_{av} versus log $\overline{M}_w$ is linear (Fig. 1), whereas the relationship between K_{av} and Stokes radius is log—log (Fig. 2). The Stokes radius of $\overline{M}_w$ of unknown sizes of dextran can be estimated using the appropriate plot.

Fig. 3 shows the elution curves obtained with serum extracts containing internal standard compared with samples containing clinical dextran demonstrating the removal of interfering substances in the region of interest. The elimination of the interfering materials in the dextran portion of the elution pattern obtained from urine is illustrated in Fig. 4. Co-precipitation of dextran with serum proteins might be expected to be dependent on the molecular size. Fig. 5 illustrates that the recovery of dextran with Stokes radii between 3 and 6 nm agrees well with expected values, although smaller and

TABLE II

CALCULATED STOKES RADII, ELUTION VOLUMES AND DISTRIBUTION COEFFICIENTS FOR THE DEFINED DEXTRAN FRACTIONS USED FOR CALIBRATION OF THE TSK (5000PW + 3000PW) DUAL-COLUMN SYSTEM

$\overline{M}_w$	Stokes radii (nm)	V_{50} ($x \pm$ 1S.D.) (ml)	K_{av}
1170	—	35.1 ± 0.3	0.83
2500	—	33.9 ± 0.4	0.77
4100	—	33.3 ± 0.4	0.71
4500	—	32.7 ± 0.4	0.71
5250	—	32.6 ± 0.3	0.71
7900	—	31.5 ± 0.3	0.65
8825	—	31.6 ± 0.2	0.66
11,500	2.48	30.9 ± 0.2	0.62
14,700	2.80	29.9 ± 0.3	0.57
21,975	3.41	29.0 ± 0.3	0.53
31,900	4.02	28.0 ± 0.3	0.48
39,200	4.40	27.4 ± 0.2	0.45
42,150	4.50	27.4 ± 0.2	0.45
73,625	5.72	25.9 ± 0.2	0.37
104,450	6.80	24.9 ± 0.2	0.32
144,960	8.10	24.3 ± 0.2	0.29
239,825	—	23.2 ± 0.2	0.24



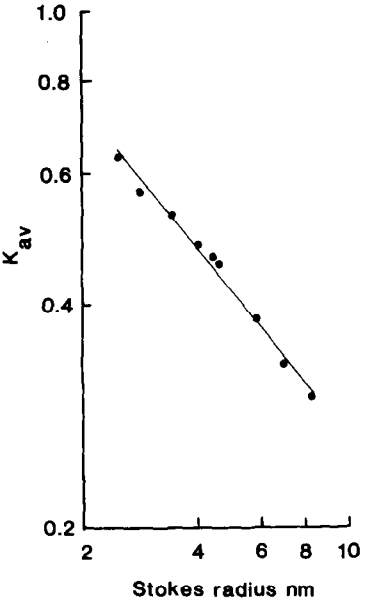


Fig. 1. Relationship between K_{av} and $\bar{M}_w$ for defined dextran fractions using the dual-column TSK (5000PW + 3000PW) system.

Fig. 2. Plot of K_{av} versus Stokes radius on log scale for defined dextran fractions using the dual-column TSK (5000PW + 3000PW) system.

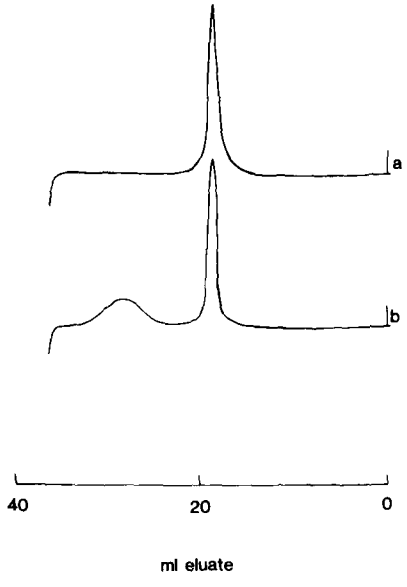


Fig. 3. Elution curves of (a) an extract of serum with internal standard but no clinical dextran; and (b) an extract of serum from a subject recently infused with clinical dextran.

larger fractions may be slightly underestimated. There is no evidence for a molecular size related variation in apparent recovery despite the use of a very-high-molecular-weight internal standard. Losses during sample preparation may be readily corrected through the incorporation of the internal standard.

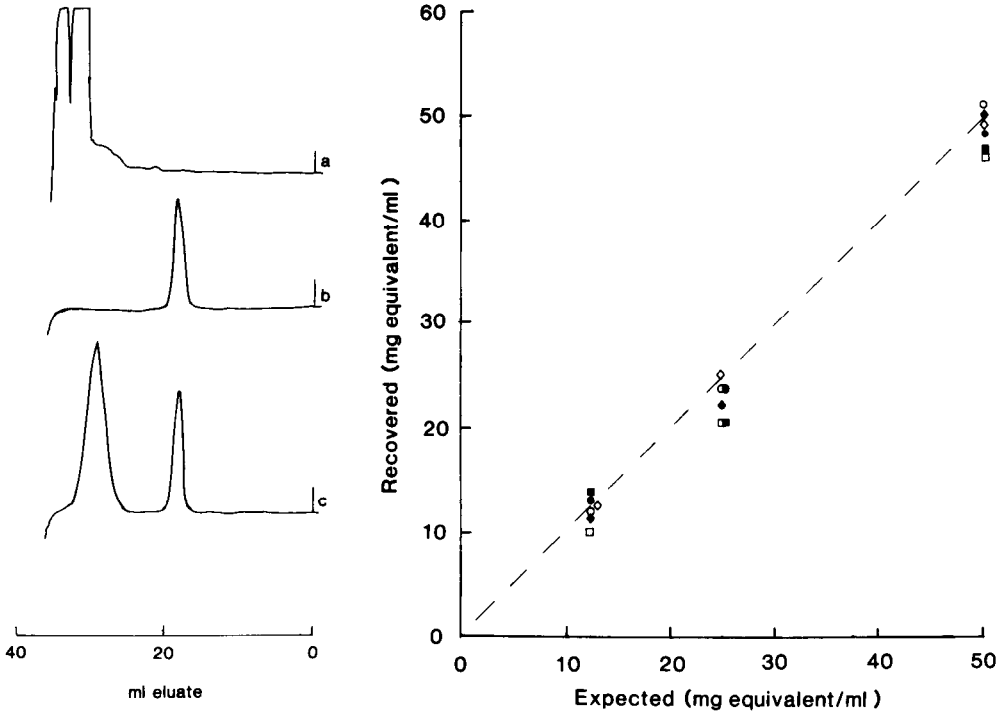


Fig. 4. Elution curves of (a) an ineffectively treated urine; (b) fully prepared urine with internal standard but containing no clinical dextran; and (c) fully prepared urine from a subject recently infused with clinical dextran.

Fig. 5. Comparison of expected and observed recoveries of known amounts of clinical dextran added to serum. The recovery for fractions of the following Stokes radii are shown: 7.2 (□), 5.7 (○), 4.6 (◇), 4.4 (●), 3.0 (◆) and 2.4 (■) nm.

Internal standard recoveries from serum ranged from 57% to 84% (mean 66%), while from urine the range was 57—67% (mean 63%). The variation in retention time of the internal standard from sample to sample was ± 0.2%.

The limit of detection of the method was determined by chromatographing a series of dilutions of dextran under standard assay conditions. The standard deviation (S.D.) of the detector response over the range of interest was calculated and a signal greater than 2 S.D. above baseline was considered significant. Under these conditions the smallest amount of clinical dextran measurable was 6 μgequiv., over the range of Stokes radii between 2.4 and 7.2 nm. This corresponds to 0.08 mgequiv./ml.

DISCUSSION

Among the intended applications of the new method was the investigation of renal glomerular function in infants and small children. This focused attention on the development of methods applicable to very small sample sizes, yet maximising the precision of the data obtained.

The measurement of the dextran components by the interference refractometer offers a substantial improvement in terms of sensitivity, labour-intensive operations, cost and time. The analysis of a single sample requires 70 min, as

compared with many hours using previously described techniques.

The volume of serum used in these studies was 0.5 ml, but as only 0.075 ml of the final preparation was loaded on to the analytical system, this sample size could be substantially reduced. Urine volumes were also amenable to reduction as only 0.050 ml of the neutralised, filtered specimens were used to charge the columns for analysis. In addition, the sensitivity of the interference refractometer could be increased 25-fold. The range of Stokes radii that can be detected is dependent upon the molecular size distribution of the polydisperse dextran preparation used. In these experiments less than 5% of the material has a Stokes radius below 2.4 nm or above 7.2 nm. Thus, the molecular size distribution of polydisperse clinical dextran infusates in the above range are readily measured using minimal sample volumes making the semi-automatic system the method of choice when studying infants or a paediatric population.

An additional advantage of the semi-automatic system described in this communication derives from the possibility of changing the exclusion limits through selection of different grades of pre-packed columns with a concomitant change of marker molecule.

Incorporation of an electronic integrator provides a continuous print-out of data which can be analysed by hand or by means of a specially written computer programme.

The development of the methodology to assay clinical dextrans in biological fluids by means of an interference refractometer depends upon an efficient sample preparation scheme. The interfering substances in both serum and urine were entirely removed using the method described above.

Limited clinical studies with healthy volunteers have yielded excellent results and will be more completely described in the clinical literature to follow.

ACKNOWLEDGEMENTS

The authors express their gratitude for the helpful and constructive discussions and assistance of Drs. Raymond Baker and Cynthia Lyne. The interest shown and data given by Dr. Ralph Gibbs of Fisons Pharmaceutical and Dr. Kirsti Granath of Pharmacia are gratefully acknowledged. The financial support of the Special Trustees of Guy's Hospital and Friends of Guy's is appreciated. Special thanks are due to Carol Switft for preparation of this manuscript.

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