EXPERIMENTAL EVALUATION OF THE ERRORS IN HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY MEASUREMENTS

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SUMMARY

A commercially available microparticulate column for high-performance gel permeation chromatography was calibrated using a number of broad dextran standards with M_n and M_w known from absolute methods. In the non-linear calibration by cubic spline, the accuracy was estimated by the mean quotient R between the polydispersity from the chromatographic measurements and the polydispersity from the known averages. From the flow-rate dependence at two temperatures, an extrapolated R value at maximum resolution was estimated to be 0.94. Comparison with a reference series of dextran standards gave a range of 0.92–0.94 for the R value, which is consistent with data from other studies. This non-physical result is due to systematic errors in the absolute methods, possibly the end-group analysis used to determine M_n. The standard deviation of 0.05 obtained for the R value is mainly determined by the random errors in the known averages. Thus the performance obtained in the chromatographic measurements with modern equipment is limited more by the errors in the absolute methods on which the calibration standards are based than by any errors inherent in the measurements.

INTRODUCTION

In gel permeation chromatography (GPC), a molecular-weight distribution *versus* elution volume is obtained for a given polymer sample. To relate the elution volume to the molecular weight, a number of polymer standards with known molecular-weight averages obtained by absolute methods are normally used under the same experimental conditions. Assuming that a calibration curve between molecular weight and elution volume can be uniquely defined for the standards used, the errors in the calibration arise both from the GPC measurements and from the measurements of the absolute molecular-weight averages. With the development of high-performance gel permeation chromatography (HPGPC), the precision of the molecular-weight averages can now been measured to 1% over the calibrtation range[1] and it is also possible, under good conditions, to obtain molecular-weight averages with an accuracy of 2% without corrections[2,3]. The molecular-weight averages as given by

manufacturers and determined by absolute methods are not always correct, and in some cases the error may be as high as 20%[4]. In view of these results, the errors in obtaining a molecular-weight distribution for a polymer sample are a result of errors more in the absolute measurements on which the calibration standards are based rather than in the GPC measurements in a good GPC system. For polymer standards with known M_n and M_w averages, an estimate of the accuracy of the polydispersity P ($P = M_w/M_n$) can be obtained. Under ideal conditions, the quotient R [$R = P(GPC)/P(ABS)$] between the polydispersity measured by GPC and the polydispersity measured by absolute methods should be equal to 1.00. Since the GPC measurements always provide a broader distribution than the real distribution, owing to the separation process, a value larger than 1.00 for this quotient is to be expected. For a number of standards covering the calibration range the mean R value should be larger than 1.00 with a random uncertainty of at least 5–10% owing to the experimental errors, mainly from the absolute measurements.

Dextran standards

The mean value of R for calibration by a number of dextran standards has been measured by the author[5] to be 1.01 ± 0.05 for the large-particle support Hydrogel, a value which is too low to be consistent with the low resolution associated with this packing material. From a study of dextran standards on silicagel by Van Dijk *et al.*[6], the mean R value for GPC measurements, both uncorrected and corrected for dispersion, can be calculated from the data in their Table I as being 1.01 ± 0.09 and 0.93 ± 0.07. Dextran standards have also been studied by Omorodion *et al.*[7] on CPG-10 glass packings. From the data in their Table 2 a mean R value can be calculated, giving 1.02 ± 0.05 for T 20 to T 150. Since a linear calibration curve has been used, the non-linear parts of the calibration curve must be excluded and T 10, T 250 and T 500 are omitted as they show unrealistic R values. In another study by Vrijbergen *et al.*[8], a mean R value can be calculated from the data in their Table II for both uncorrected and corrected GPC measurements of the dextran standards T 20 to T 500 in water on Porasil, giving 1.05 ± 0.11 and 0.99 ± 0.11, respectively. As these values differ from those obtained in other studies, and also have a comparatively high standard deviation, they must be regarded as less certain. Thus the studies on large-particle support give R values of nearly 1.00 for GPC measurements uncorrected for dispersion and a value lower than 1.00 for corrected GPC measurements. Even if the random uncertainty in the absolute methods used for the dextran standards, such as light-scattering and end-group analysis, are as high as 4.8% for M_n and 2.9% for M_w[5,9] and consistent with the standard deviation of 5% for the quotient, it can still not explain the consistently low value obtained for R. An experimental evaluation has therefore been carried out in order to determine the accuracy in the calibration by dextran standards on small-particle supports for HPGPC. Under such conditions the errors in the GPC measurements can be made sufficiently small to allow a more accurate measure of R.

EXPERIMENTAL

GPC procedure

The HPGPC equipment and method used in this study were essentially the

same as described earlier[1]. Degassed solvent, 0.5 mM sulphuric acid solution, was used at flow-rates from 0.2 to 0.8 ml/min. The samples, dextran standards (Pharmacia, Uppsala, Sweden) with a concentration of 1.5 μg/μl, were injected with a Rheodyne Model 7010 injection valve with a 10-μl sample loop. The sample amount was chosen as low as 15 μg for a total elution volume of 6 ml, to minimize concentration effects. For the HPGPC measurements, two E-linear μBondagel columns (Waters Assoc.) and a Multiref 901 (Optilab Instrumentation, Vällingby, Sweden) refractive index detector with a 10-mm measuring cell were used. The Multiref detector was chosen because of its high sensitivity. For a detector time constant of 0.3 sec the standard deviation of the baseline noise was measured to be less than $3 \cdot 10^{-9}$ refractive index units at a flow-rate of 0.2 ml/min. The columns and detector cell were kept at the same temperature. Time-based HPGPC measurements were made at both 30 and 70°C.

The choice of model for the calibration curve has been evaluated by the author in an earlier paper[5]. The cubic spline model was found to be the best representation of the calibration curve with the limited data available for the calibration, and it was also used in this study. Calibrations with known M_n and/or M_w have also been evaluated earlier[5], and the differences were found to be insignificant. Since the accuracy increases with the number of known averages used in the calibration, both M_n and M_w are used in the calibration. For the HPGPC measurements, the standards were run in random order. Unbiased and accurate estimates of the molecular-weight averages were obtained by determining the baseline of the GPC curve by computer. The effects of a non-linear detector response[10] and a detector response that varied with molecular weight[11] were measured and found negligible.

Dextran standards

Seven broad dextran standards (Pharmacia) were used for the calibration. Another series of six broad dextran standards by Pharmacia, available from the WHO Collaboration Centre for Chemical Reference Substances (Apoteksbolaget, Sweden), were also used for comparison. The M_n values of the standards were determined by end-group analysis using the Somogyi copper phosphate method and M_w values by light-scattering measurements[9].

TABLE I

ABSOLUTE MOLECULAR-WEIGHT AVERAGES OF THE TWO SERIES OF DEXTRAN STANDARDS

The figures in parentheses indicate the number of measurements.

Dextran standard	Pharmacia		Apoteksbolaget	
	M_n	M_w	M_n	M_w
T10	6400	10 500	5870 ± 195(7)	10 210 ± 260(7)
T20	16 700	21 600	16 980 ± 475(7)	21 640 ± 460(7)
T40	29 500	39 500	26 920 ± 1255(6)	39 690 ± 380(7)
T70	42 500	70 000	41 170 ± 1060(7)	69 980 ± 1060(8)
T110	74 000	105 000	–	–
T150	85 000	143 000	87 460 ± 2440(7)	144 080 ± 1945(9)
T250	106 500	268 000	113 970 ± 4200(7)	240 570 ± 3865(7)

RESULTS AND DISCUSSION

Flow-rate dependence
    The quotient $R = P(GPC)/P(ABS)$ has been calculated from the GPC cali-
bration at flow-rates from 0.2 to 0.8 ml/min (0.028 to 0.112 cm/sec) and at two
temperatures, 30 and 70°C. Fig. 1 indicates that the flow-rate has little effect on the
R value in this range. The data at higher flow-rates are somewhat uncertain owing
to the higher pulsation noise from the pump and the high sensitivity of the detector.
The R values are lower for the runs at the higher temperature, and this difference
decreases with the flow-rate. A cautious extrapolation to maximum resolution, which
is near zero flow-rate, gives an approximate limit for R as 0.94 at both temperatures.
With the standard deviation of R, which varies over the flow-rate range from 0.04
to 0.05, the R value at near-zero flow-rate can be estimated to be 0.94 ± 0.05. This
is in good agreement with the R value calculated from the data taken by Van Dijk
et al.[6] for dispersion-corrected GPC measurements,, 0.93 ± 0.07. The value taken
from the data by Vrijbergen *et al.*[8] for corrected GPC measurements, 0.99 ± 0.11,
is more uncertain, since actual HPGPC measurements give lower R values. With both
uncorrected and corrected GPC measurements that give higher R values than other
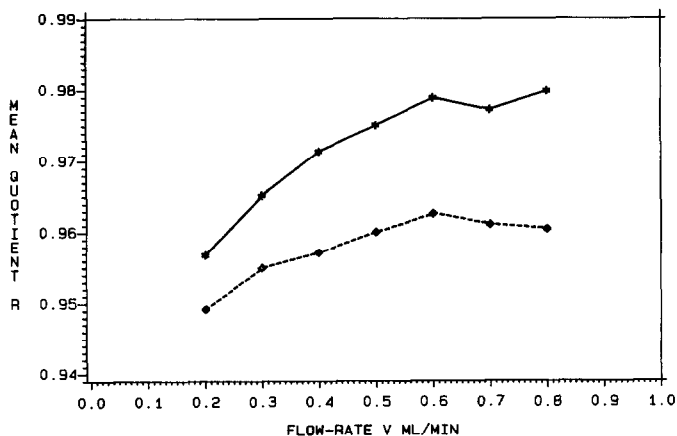studies, it must be concluded that their GPC procedure is not correct.

Fig. 1. Flow-rate dependency of the mean quotient R for dextran standards from Pharmacia. The upper
trace refers to measurements at 30°C and the lower trace to measurements at 70°C.

    The extrapolated R value of 0.94 is perhaps still too large, since the detector
cell with connecting tubing introduces a noticeable tailing at the low-molecular-
weight end of a polymer sample, even in this low flow-rate range, and has therefore
perhaps not been properly taken into account in the extrapolation to zero flow-rate.
In a study by Rand and Mukherji[12], two HPGPC columns with microparticulate
packings were compared for efficiency and reproducibility. The authors have found
that Bimodal II columns (DuPont) have a superior resolution to the E-linear μBon-
dagel columns (Waters) used in this study. Most of this difference could be predicted
because of the larger volume in Bimodal columns with the consequent lesser effect
of band broadening from injection and detector volume. The larger volume also

makes a comparison unfavorable to μBondagel columns at the same flow-rate. Even so, their data[12] indicate that Bimodal columns have an intrinsically higher perform-ance, and it is probable that a somewhat lower extrapolated R value could be found for these columns. Thus the R value of 0.94 should not be considered as an accurate measure of R but rather as an upper limit for this value.

Calibration standards

The extrapolated R value of 0.94 is associated with a standard deviation of 0.05, a value consistent with the value 0.05 obtained earlier on a large-particle sup-port[5]. With a standard deviation for the absolute methods of 4.8% for M_n and 2.9% for M_w[5,9], the precision of the quotient R will be determined by the uncertainty in the known averages. The choice of well-characterized dextran standards is therefore of fundamental importance for an accurate R value. A series of well-characterized dextran references standards available from Apoteksbolaget has been used to evalu-ate the accuracy in the R value. To compare the two series of dextran standards with the same number of standards, T 110 was omitted. At a flow-rate of 0.2 ml/min, the R values are 0.96 ± 0.05 (Pharmacia) and 0.94 ± 0.05 (Apoteksbolaget). Thus the well-characterized reference series has an R value that is 2% lower than the series from Pharmacia and the extrapolated R value should therefore probably be 0.92 rather than 0.94. For both series, the extrapolated R values are in the range 0.92–0.94 ± 0.05 on a small-particle support, which is consistent with the value of 0.93 ± 0.07 calculated from the dispersion-corrected data by Van Dijk *et al.*[6] on a large-particle support.

Absolute methods

To estimate the errors in the absolute methods, a comparison between different methods can be used. In the study of Van Dijk *et al.*[13], the M_n values of dextran standards were also measured by osmometry. From the data in their Table I a quo-tient $M_n(EGA)/M_n(OSM)$ between the M_n given by Pharmacia for the end-group analysis and the M_n measured by osmometry can be calculated. The quotient ob-tained in this way is 0.96 ± 0.08. Even if the uncertainty is large compared with the deviation from 1.00, it is interesting to note that if osmometry had been used instead of end-group analysis, the quotient R between the polydispersity measured by GPC and polydispersity measured by absolute methods would have approached 1.00. Thus the low value of R might be explained by systematic errors in the absolute methods.

CONCLUSIONS

Under good experimental conditions, the precision in the calibration is almost completely determined by the precision in the known averages. With a number of calibration standards or known molecular-weight averages that is larger than the number of parameters in the calibration curve representation, a least-squares esti-mation of the overdetermined system can reduce the effects of the random errors in the known averages. The larger number of standards can also provide a more ac-curate non-linear representation of the calibration curve. The common use of a linear calibration curve based on two known molecular-weight averages does not take these major effects into account, and therefore such a calibration procedure does not use the performance inherent in the HPGPC measurements.

Dispersion has been considered as an important error in GPC analysis. With modern GPC equipment this error can be minimized under suitable experimental conditions, and the remaining error can be ignored in comparison with errors in the absolute determined averages. For an even more correct result the GPC curves can be corrected for the dispersion if monodisperse calibration standards are available.

If there are any systematic errors in the determination of the molecular-weight averages by absolute methods, these errors will also be present in the calibration curve, since the GPC method is not an absolute method. For the dextran standards it is shown that for both large- and small-particle supports, the polydispersity measured by GPC is lower than the polydispersity measured by absolute methods. This is a non-physical result, which must be due to systematic errors in the absolute methods on which the calibration standards are based, since no errors in the GPC measurements have been found that can account for this result. GPC measurement in itself cannot provide any information on the absolute method or methods that have systematic errors associated with it. However, a comparison of the results for different absolute methods could provide some insight. Data for the two methods, end-group analysis and osmometry, for M_n at least indicate that the low R values obtained can be explained by systematic errors in the method of end-group analysis.

Thus the careful characterization of polymer samples for use in the calibration of the GPC separation is of vital importance, if the performance of HPGPC is to be obtained. It has even been stated by Billmeyer, "More use is being made of empirical but rapid techniques such as GPC, but less regard is being paid to the need for the calibration samples required to give them meaning"[14].

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