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Note

Gas–liquid chromatographic assay of D-ribose and its per(trimethylsilyl)ated oxime

RICHARD J. STROZ

Quality Control Department, Hoffmann-La Roche Inc., Belvidere, New Jersey 07823 (U.S.A.)

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A major problem in the application of gas–liquid chromatography (g.l.c.) to sugar analysis is that many compounds give rise to multiple peaks. These peaks are those of structurally different anomers formed during the preparation of derivatives. Acyclic derivatives have been successfully employed to lessen the number of peaks from each sugar. Sweeley *et al.*[1] introduced the use of the oximes, acyclic sugar derivatives amenable to per(trimethylsilyl)ation; this work was then extended to structural analysis by g.l.c.–mass spectrometry[2–5], and analysis of disaccharides[6]. Preliminary quantitation of sugars analyzed as per(trimethylsilyl)ated oximes has been performed[7], but no in-depth, quantitative investigation of sugars has been attempted using these derivatives. In the present work, aqueous solutions of D-ribose were extensively examined by use of the oximation–(trimethylsilyl)ation procedure.

Derivatization of unsubstituted sugars is a necessary prerequisite to analysis by g.l.c., because of their polar, nonvolatile nature. Since the introduction of the use of trimethylsilyl ethers of carbohydrates by Sweeley *et al.*[1], this technique has become very common. The analysis of such derivatives is often complicated by formation of multiple peaks caused by various structural and anomeric forms of each sugar. This disadvantage has been circumvented by first converting the sugar into an acyclic derivative such as the alditol or aldononitrile, before formation of a volatile derivative. By combining the ability of oximation to produce acyclic compounds with that of (trimethylsilyl)ation to afford volatile ethers, quantitation of D-ribose as a single peak in an aqueous medium, with concomitant separation from D-glucose, D-gluconic acid, and ribitol was achieved in the present work.

The oxime was formed by reaction of D-ribose with hydroxylamine hydrochloride in pyridine by a procedure comparable to that described previously[1]. Although a reaction time of 30 min was employed for quantitative purposes throughout this work, use of a reaction time of 5 min was also examined. Judging both qualitatively [one peak formed after (trimethylsilyl)ation] and quantitatively (reasonable correction factors and assay values), the time allowed for oximation can probably be lessened to 5 min.

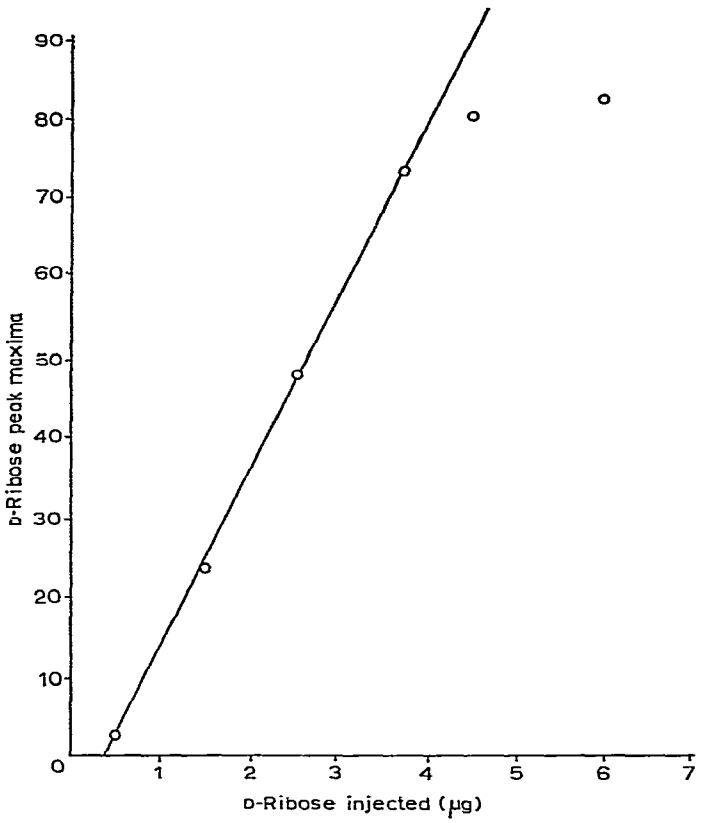

Fig. 1. Examination of detector response *vs.* amount of D-ribose injected [as the per(trimethylsilyl)ated oxime derivative].

The (trimethylsilyl)ation was essentially instantaneous, presumably because, besides rendering the sugar acyclic, oximation causes hydroxyl groups previously sterically hindered to become more amenable to (trimethylsilyl)ation. The white precipitate formed upon addition of the (trimethylsilyl)ation reagents, presumably ammonium chloride, had no apparent effect upon the analyses or the equipment. It was also found convenient that both the oximation and the (trimethylsilyl)ation steps were completed using the same solvent, namely, pyridine. Besides being an excellent solvent for both reactions, pyridine neutralizes the hydrochloric acid liberated. No isolation of the oxime, or removal of the excess of hydroxylamine, was attempted, and no detrimental effects resulted. The etherification described can tolerate a small proportion of water. Previous work[8] had shown that 40 mg of water could be tolerated, using a different (trimethylsilyl)ation mixture. During the course of these experiments, up to 30 mg of water (see Experimental section) had no deleterious effects.

The linearity of the detector response for D-ribose is given in Fig. 1. For injections of 0.5 to 3.75 μg of D-ribose, the detector response is linear; however, at

TABLE I

STUDY OF RECOVERY OF KNOWN QUANTITIES OF D-RIBOSE, DETERMINED BY G.L.C. ASSAY AS THE PER(TRIMETHYLSILYL)ATED OXIME DERIVATIVE

Ribose [% (w/w)]		*Difference (%)*
Theoretical	*Experimental*	
10.3	10.4	1.0
12.1	12.2	0.8
25.2	24.6	2.4
25.7	25.4	1.2
51.1	51.3	0.4
51.6	52.5	1.7
74.7	75.8	1.5
74.4	74.4	0.0
88.4	88.2	0.2
88.6	87.1	1.7

TABLE II

REPRODUCIBILITY OF MULTIPLE INJECTIONS OF A SAMPLE CONTAINING A KNOWN WEIGHT OF D-RIBOSE. ASSAY OF D-RIBOSE[a] DETERMINED BY G.L.C. AS THE PER(TRIMETHYLSILYL)ATED OXIME DERIVATIVE

Injection No.	*Found D-ribose [% (w/w)]*	*Difference from theoretical (%)*
1	88.8	0.5
2	88.4	0.0
3	89.0	0.7
4	89.8	0.5
5	89.4	1.1
6	88.3	0.1
7	88.3	0.1
8	87.5	1.0
9	88.0	0.5
10	87.5	1.0
11	87.7	0.8
12	87.9	0.6

[a]Theoretical % (w/w) of D-ribose, 88.4%.

injection of 4.5 μg, linearity is not continuous with other points. For all quantitative investigations, samples were so adjusted that the linear range of D-ribose was not exceeded. The recovery of known amounts of D-ribose is given in Table I. The experimental values for the % (w/w) of D-ribose were determined by g.l.c. assay by using the procedure described here. With one exception, all assays lay within the generally accepted 2% error for g.l.c. analyses. The reproducibility of multiple injections of the same sample is shown in Table II. No difference greater than 1.1% was found, and the results are acceptable. The reproducibility of the assay of multiple prepara-

TABLE III

G.L.C. ASSAY OF SAMPLES CONTAINING AUTHENTIC D-RIBOSE, AS THE PER(TRIMETHYLSILYL)ATED OXIME DERIVATIVE

Sample	*Ribitol* % *(w/w)*	D-*Ribose* % *(w/w)*	D-*Ribose average* % *(w/w)*	*Standard deviation*
RS-110A-2	0.4, 0.4, 0.4, 0.4 0.4, 0.4, 0.4, 0.4	27.2, 27.4, 27.6, 27.4 27.1, 27.4, 27.7, 27.6	27.4	0.2
RS-111	0.4, 0.4, 0.4, 0.4 0.4, 0.4, 0.4, 0.4	21.1, 21.0, 20.7, 20.7 20.8, 20.8, 21.1, 21.0	20.9	0.2
RS-111A	0.8, 0.7, 0.8, 0.7 0.9, 0.9, 0.8, 0.8	48.5, 48.8, 48.4, 47.7 47.9, 48.3, 48.2, 48.3	48.3	0.3
RS-111E	0.9, 0.8, 0.9, 0.9 0.8, 0.8, 0.8, 0.8	51.3[a], 50.3, 50.4, 50.1 49.4, 49.4, 50.2, 50.6	50.1	0.5
RS-4811	0.7, 1.0, 0.8, 0.9 0.9, 0.9, 0.9, 0.9	67.2, 68.1, 67.5, 67.7 67.4, 67.6, 66.9, 67.4	67.5	0.4
RS-4812	0.7, 0.9, 1.0, 0.9 0.9, 0.9, 0.8, 0.9	64.0, 64.1, 63.9, 64.2 63.9, 64.0, 63.8, 64.3	64.0	0.2

[a]Assay value not included in the calculation for average or standard deviation. The value is a statistical outlier in the 95% confidence level, as determined by the *t*-test method.

TABLE IV

COMPARISON OF ASSAYS OF D-RIBOSE BY THE ORCINOL SPECTROPHOTOMETRIC METHOD AND BY G.L.C. OF THE PER(TRIMETHYLSILYL)ATED OXIME DERIVATIVE

Sample	*Orcinol assay* (% *w/v*)	*G.l.c. assay* (% *w/v*) *(in triplicate)*
1	0.2	0.02, —, —
2	0.3	0.1, 0.1, 0.1
3	0.5	0.3, 0.3, 0.3
4	1.0	0.7, 0.7, 0.7
5	1.9	1.6, 1.6, 1.6
6	3.4	2.9, 2.9, 3.0
7	5.3	4.7, 4.9, 4.9
8	6.3	6.0, 6.0, 6.1
9	7.3	6.9, 6.9, 6.9
10	7.4	7.0, 6.9, 6.9
11	7.2	7.0, 7.1, 7.0

tions of samples containing various proportions of D-ribose is given in Table III. Good reproducibility between preparations is evident.

A series of experiments was conducted to evaluate the traditional methods for the assay of D-ribose, namely, the orcinol spectrophotometric method and the Fehling titration method, *versus* the oximation–(trimethylsilyl)ation–g.l.c. assay. In general, the results show a lower assay by the g.l.c. procedure (see Tables IV and V);

TABLE V

COMPARISON OF ASSAYS OF D-RIBOSE USING THE FEHLING TITRATION METHOD AND G.L.C. OF THE PER(TRIMETHYLSILYL)ATED OXIME DERIVATIVE

Sample	*Fehling titration assay (% w/w) (in duplicate)*	*G.l.c. assay (% w/w) (in duplicate)*
033	65.6, 65.9	65.6, 65.2
034	66.4, 66.2	67.3, 67.3
035	67.2, 66.8	66.7, 66.2
036	66.8, 67.4	65.9, 65.6
037	67.1, 67.5	65.7, 65.9
038	66.5, 65.5	62.8, 62.2
039	67.0, 66.8	66 2, 66.2

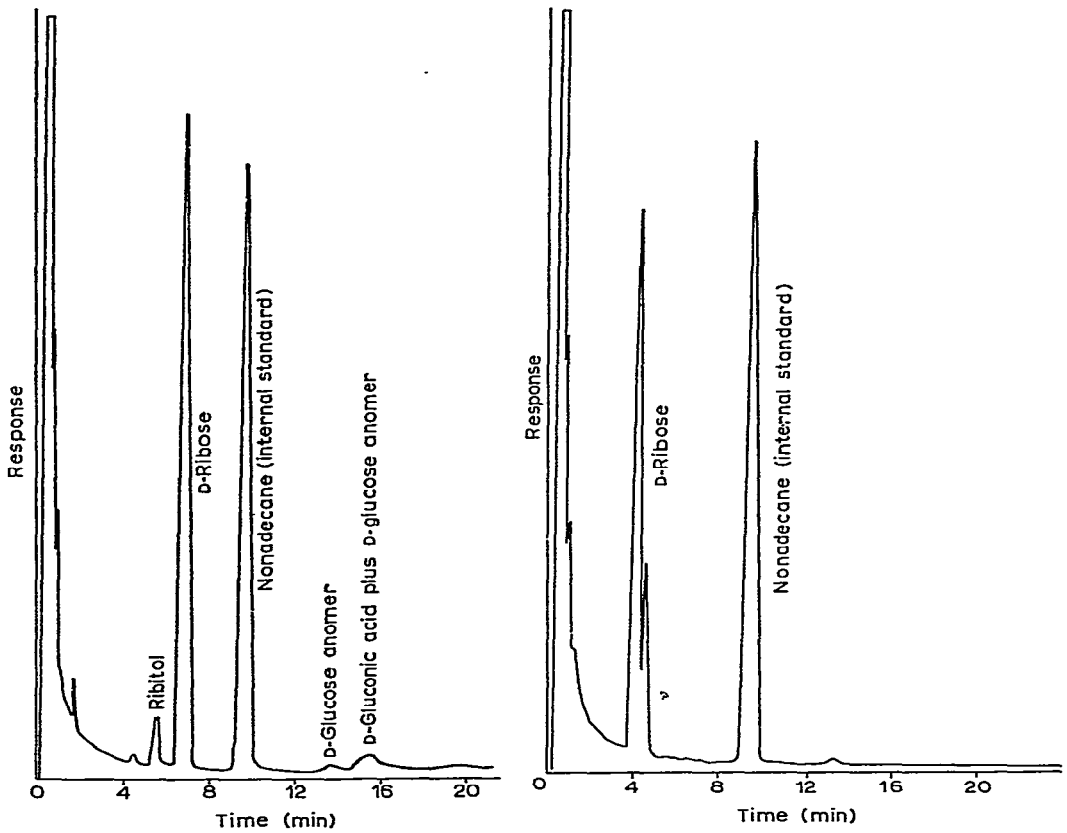

Fig. 2. G.l.c. separation of D-ribose standard from standards of ribitol, D-glucose, and D-gluconic acid as the per(trimethylsilyl)ated oxime derivatives, with nonadecane as the internal standard; chromatographed on a column of 10% of SE-52, using a flame-ionization detector.

Fig. 3. G.l.c. separation of per(trimethylsilyl)ated D-ribose standard from nonadecane (internal standard); chromatographed on a column of 10% of SE-52, using a flame-ionization detector.

this was to be expected, as neither the orcinol spectrophotometric method nor the Fehling titration method is specific for D-ribose; they are general tests for pentoses and reducing sugars, respectively. The g.l.c. procedure was developed as a specific assay for ribose.

A graph for a four-component standard containing D-ribose, ribitol, D-glucose, and D-gluconic acid, in addition to the internal standard, is given in Fig. 2. The difference between the per(trimethylsilyl)ated D-ribose oxime and per(trimethylsilyl)ated D-ribose is seen in Figs. 2 and 3. That the peak for D-ribose in Fig. 2 is a single peak, and not multiple peaks combined under one peak, can be stated with some certainty, because, besides the column of SE-52 used for the analytical work, columns of 10% of OV-17 and of 10% of XE-60 were employed to determine whether other peaks could be separated from the peak for D-ribose. Both packings have polarity higher than that of SE-52, XE-60 being the most polar.

A single, Gaussian peak was obtained when the per(trimethylsilyl)ated D-ribose oxime was chromatographed on any of the three columns.

Figs. 4 and 5 show the chromatography of authentic samples of D-ribose on 10%

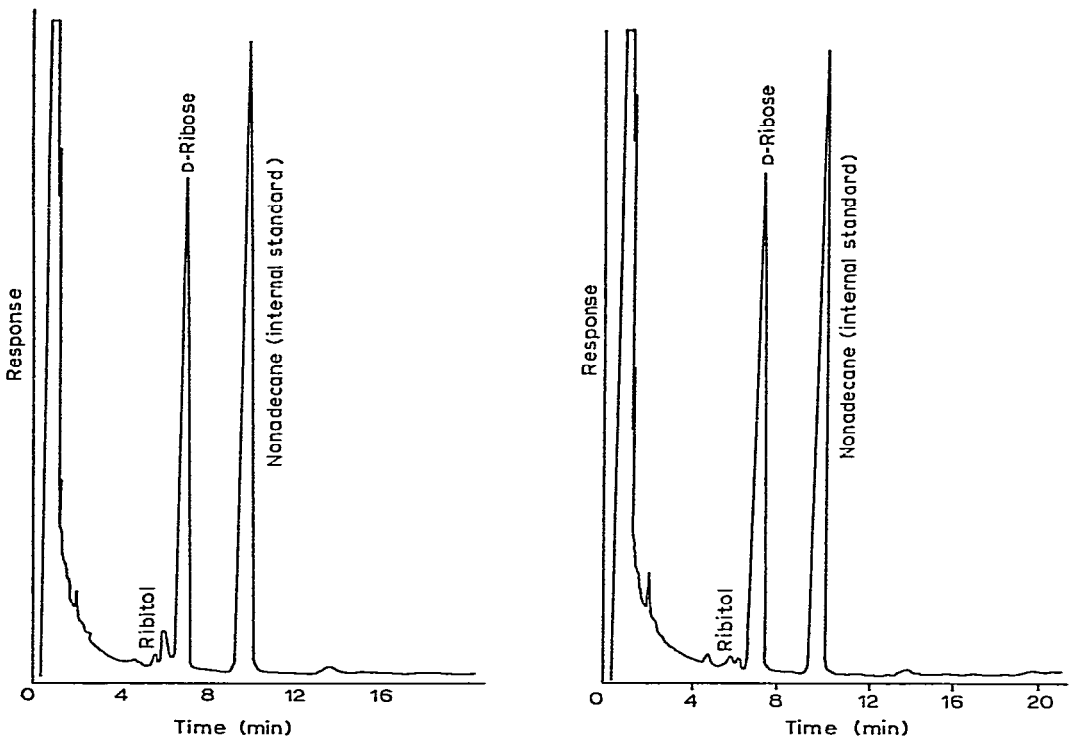

Fig. 4. D-Ribose (sample RS-110A-2) derivatized by oximation and then per(trimethylsilyl)ation; chromatographed on a column of 10% of SE-52, using a flame-ionization detector.

Fig. 5. D-Ribose (sample RS-4811) derivatized by oximation and then per(trimethylsilyl)ation; chromatographed on a column of 10% of SE-52, using a flame-ionization detector.

of SE-52. For the sake of brevity, only two graphs of samples are included, as Fig. 4, that of RS-110A-2, is typical of samples RS-111, RS-111A, and RS-111E. Likewise, Fig. 5 is typical of the chromatography of RS-4812, as well as of that of RS-4811. Although not baseline-separated from the unknown compounds eluted close to it, the ribitol peak in Fig. 4 proved no problem as far as reproducible assays were concerned (see Table III).

D-Glucose and D-gluconic acid were not extensively examined. In agreement with the results of a previous study[5], D-gluconic acid was found to be only slightly soluble in pyridine; therefore, attainment of complete, quantitative derivatization was questionable. Derivatization of D-glucose resulted in formation of two peaks, one of which could not be separated on the SE-52 column from that of D-gluconic acid (see Fig. 2). In subsequent investigations, it was found that the XE-60 column is capable of separating all three peaks. As there are many other methods for the determination of D-glucose (*e.g.*, enzymic) and D-gluconic acid (*e.g.*, the ferric chloride spectrophotometric method) that are sensitive enough for use in low-level assays, this matter was not pursued. However, it was important for the purpose of assay of D-ribose that sufficient separation from these possible contaminants was ensured.

In conclusion, it is considered that this method present a facile, rapid procedure for the derivatization and assay of aqueous solutions of D-ribose. Adaptation to crystalline samples should pose no difficulties. Preliminary attempts at quantitation using the oximation–(trimethylsilyl)ation procedure for sugars have been reported[7], but the present work constitutes the first in-depth, quantitative determination of D-ribose by this derivatization technique.

EXPERIMENTAL

Gas–liquid chromatography. — This was performed with a Hewlett–Packard Model 5730 gas–liquid chromatograph equipped with a hydrogen-flame ionization detector, and a stainless-steel column (1.83 m × 3.17 mm o.d.) fitted for on-column injection and packed with 10% of SE-52 (5% phenyl, silicone gum) on Gas Chrom Q (80–100 mesh), with nitrogen at 20 $mL.min^{-1}$ as the carrier gas, and hydrogen at 30 $mL.min^{-1}$ and air at 300 $mL.min^{-1}$ for the detector gases. The operating temperatures were: detector, 300°; injector, 250°; and column, 203°. The injection volume was 2 μL, the electrometer range was × 10, the attenuation was × 128, the recorder was a Hewlett–Packard 7123A instrument operated at a chart speed of 6.35 $mm.min^{-1}$, and the integration and calculations were electronic (Hewlett–Packard 3351A Lab Data System).

Reagents. — Hydroxylamine hydrochloride, D-glucose, and pyridine were certified ACS grade (Fisher Chemical Company, Springfield, NJ); hexamethyldisilazane and chlorotrimethylsilane of silylation grade and SE-52 stationary phase were obtained from Analabs, Inc., North Haven, CT; D-ribose, ribitol, sodium D-gluconate (98%), and nonadecane were obtained from Sigma Chemical Company, St. Louis, MO; Gas Chrom Q (80–100 mesh) was from Applied Science, Inc., State

College, PA; the glass vials (15-mL capacity, with vinyl-lined screw-caps) were obtained from Ace Scientific Supply Co., Inc., Linden, NJ.

Preparation of standards and samples. — *D-Ribose stock solution.* Accurately weight 3.00 g of D-ribose into a 10-mL volumetric flask. Dissolve in water, and dilute to volume with water.

Ribitol stock solution. Accurately weigh 3.00 g of ribitol into a 10-mL volumetric flask. Dissolve in water, dilute to volume with water, and dilute 1:20 with water.

Internal-standard solution. Accurately weigh 900 mg of nonadecane into a 100-mL volumetric flask. Dissolve in pyridine, and dilute to volume with pyridine.

Standard, working solution. Place 40 μL each of the D-ribose and ribitol stock solution in a glass vial. This is equivalent to 12.00 mg of D-ribose and 0.60 mg of ribitol. Azeotrope the water with a few drops of methanol, and evaporate to dryness on a steam bath under a stream of nitrogen. Add 2 mL of hydroxylamine hydrochloride in pyridine (25 mg/mL), stopper, and keep for 30 min in an oven at 70–75°. Per(trimethylsilyl)ate with hexamethyldisilazane (2 mL) and chlorotrimethylsilane (1 mL). Add nonadecane (2 mL) as the internal standard, and then pyridine (3 mL). Shake the vial to mix the reagents, and inject the supernatant liquor directly.

Working solution of sample. Accurately weigh into a glass vial an equivalent volume of D-ribose solution to contain an amount of D-ribose approximately equal to that of the standard. Azeotrope with methanol, if necessary, and then proceed as per "Standard, working solution".

Equations

$$\text{Detector response ratio (DRR)} = \frac{(\text{area of nonadecane}) \times [\text{weight of standard (mg)}]}{(\text{area of standard}) \times [\text{weight of nonadecane (mg)}]}$$

$$\%\ (\text{w/w}) \text{ of component} = \frac{(\text{area of component}) \times [\text{weight of nonadecane (mg)}] \times \text{DRR} \times 100}{(\text{area of nonadecane}) \times [\text{weight of sample (mg)}]}$$

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