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Note

Direct spectrophotometric thin-layer chromatography for determining malondialdehyde and β-formyl pyruvate produced by the periodate oxidation of some deoxy sugars

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The sensitive periodate–thiobarbituric acid reaction is widely used for the quantitative determination of deoxy sugars such as 2-deoxyribose[1], 3,6-dideoxyhexoses[2] and 2-keto-3-deoxy-onic[3,4] and sialic acids[5,6], all of which are structural components of numerous biologically important compounds, *e.g.*, glycoproteins, glycolipids, bacterial lipopolysaccharides, certain hormones and enzymes.

Preparations of the above compounds often contain a mixture of thiobarbituric acid chromogens, namely malondialdehyde and *β*-formyl pyruvate, which are formed by the periodate oxidation of the above neutral and acidic deoxy sugars, respectively. The simultane-ous determination of these chromogens by spectrophotometric methods is difficult[7–10].

In this paper, we suggest a quantitative thin-layer chromatographic method for determining malondialdehyde and *β*-formyl pyruvate that will be useful as the final stage of analysis for numerous biologically important compounds.

Chromatography should be carried out subsequent to hydrolysis and periodate oxidation, the conditions of which should take account of the specific structures and properties of the substances being studied.

We have applied our method of bacterial lipopolysaccharides (LPS) containing 2-keto-3-deoxyoctonic acid (KDO) and 3,6-dideoxyhexoses (3,6-DDH) as structural components.

EXPERIMENTAL

Materials and apparatus

KDO ammonium salt (monohydrate) and 3,6-dideoxy-D-glucose (paratose) were synthesized and purified according to Chalambor *et al.*[11] and Ekborg and Svensson[12].

The LPS preparation was isolated from gram-negative bacteria of *Yersinia pseudotuberculosis* and characterized as described earlier[13].

All other reagents and solvents used were of analytical-reagent grade.

The solvent system used in thin-layer chromatography (TLC) was chloroform–acetone (1:2). TLC plates (5 × 7.5 cm) coated with silica gel on aluminium foil (Silufol, Kavalier, Sklárny, Czechoslovakia) were used.

A Shimadzu Model CS-900 dual-wavelength TLC scanner was used.

Calibration graph

To determine the calibration coefficients, five standard solutions of KDO and 3,6-DDH of various concentrations and weight ratios were used (see Table I). The concentrations of KDO (2–20 μg per 0.2 ml) and 3,6-DDH (1–5 μg per 0.2 ml) were selected according to their contents in known preparations of bacterial LPS. Aliquots (0.2 ml) of each standard solution were taken at all stages of the analytical procedure described below, with the exception of hydrolysis.

TABLE I

STANDARD SOLUTIONS OF KDO AND 3,6-DDH USED FOR CALIBRATION

Solution No.	*Amount (μg per 0.2 ml)*		*Weight ratios*	
	KDO	*3,6-DDH*	*KDO:3,6-DDH*	*3,6-DDH:KDO*
1	19.60	0.89	22.022	0.045
2	13.06	2.36	5.533	0.180
3	10.80	2.54	4.251	0.235
4	5.73	2.80	2.046	0.488
5	2.33	4.50	0.571	1.931

Procedure

The LPS preparation was hydrolyzed under conditions described previously[14].

Two aliquots (0.1 ml) of an LPS hydrolyzate were taken; water (0.1 ml) was added to the first and an aqueous solution of KDO (0.1 ml), concentration 100–150 μg/ml, to the second aliquot in separate test-tubes. Subsequently, 0.2 ml of sodium periodate solution (0.125 *M* in 0.5 *N* sulphuric acid) was added to the contents of each test-tube and oxidation was carried out for 10 min at 55°. After cooling, the excess of periodate was degraded by adding 0.4 ml of sodium arsenite solution (8% in 0.5 *N* hydrochloric acid). A colour reaction was performed by adding 2.5 ml of 2-thiobarbituric acid solution (0.6% in water), heating for 10 min on a boiling water-bath and then cooling; subsequently, sodium sulphate (1 g) and cyclohexanone (0.5 ml) were added. The mixture was shaken violently and then centrifuged. A major portion of the extract obtained was transferred into a small test-tube containing anhydrous sodium sulphate.

Chromatography

The extract (2 μl) was applied to the starting line, which was 1.5 cm from the edge of the plates. Chromatography was carried out by placing the plates in a glass chamber (10 × 20 × 20 cm) containing chloroform–acetone (1:2) solvent (5 ml) at room temperature. The developed plates were removed from the chamber and dried in air.

The peak areas of the individual spots were determined by the linear scanning

method in the direction of flow of the solvent through the centre of each spot, using a sensitivity setting of 5, a scanning rate of 30 mm/min. A wavelength of 550 nm was employed for the sample and the reference wavelength was 700 nm. Peak areas were calculated as the product of the peak height and the width at half-height.

RESULTS AND DISCUSSION

The structures and properties of the coloured compounds formed by the reaction of malondialdehyde and β-formyl pyruvate with 2-thiobarbituric acid were examined by Kuhn and Lutz[15], who noted the good stability of the chromophores in water at acidic and neutral pH and in organic solvents. Our experiments showed that these chromophores possess good chromatographic properties: the substances had an intense red-crimson colour, which remained on the TLC plates for several days. The substances are readily extracted from the reaction medium into cyclohexanone (the degree of extraction being as much as 98.7%), and can thus be concentrated in small volumes.

The fact that these compounds have different polarities makes it possible to separate them accurately on a thin layer of silica gel. The $R_F \times 100$ values of malondialdehyde and β-formyl pyruvate chromophores in the chloroform–acetone (1:2) solvent system were 40 and 12, respectively.

The production of coloured compounds *in vitro* and their subsequent chromatography enabled us to avoid several difficulties connected with performing a quantitative reaction on chromatographic plates. Difficulties in selecting an internal standard were avoided by using the method of relative calibration, in which the internal standard is one of the substances to be determined[16]. For the LPS preparation, either KDO or 3,6-DDH may serve as the internal standard; in our work, KDO was used.

Relative calibration coefficients were determined using five chromatoplates, on to each of which five calibration solutions were applied (see Table I). The results are given in Table II.

TABLE II

DETERMINATION OF CALIBRATION COEFFICIENTS (*C*) FOR KDO AND 3,6-DDH
Standard deviation, 1.33%. Confidence interval with a confidence probability of $P = 0.95$ and 9 degrees of freedom, 2.38%.

Area ratio, KDO:3,6-DDH	*Weight ratio, KDO:3,6-DDH*	C_{KDO}	*Area ratio, 3,6-DDH:KDO*	*Weight ratio, 3,6-DDH:KDO*	$C_{3,6\text{-DDH}}$
2.298	22.022	0.104 (5)*	0.436	0.045	9.609 (5)*
0.607	5.533	0.109 (5)	1.647	0.180	9.150 (5)
0.472	4.251	0.111 (5)	2.116	0.235	9.004 (5)
0.232	2.046	0.113 (5)	4.312	0.488	8.836 (5)
0.058	0.517	0.112 (5)	17.207	1.931	8.910 (5)
		Mean ($n = 25$) 0.109			Mean ($n = 25$) 9.100

* Number of determinations.

It can be seen from Table II, the calibration coefficients between 2–20 ng of KDO and 1–5 ng of 3,6-DDH per spot are constant, with mean values of 0.109 and 9.100 for KDO and 3,6-DDH, respectively. The standard deviation calculated from 25 chromatograms (five TLC plates each with five chromatograms) was 1.33%, the confidence interval with a confidence probability of $P = 0.95$ for 10 chromatograms being 2.38%.

If one takes into consideration that for routine analyses the reproducibility of the determination may be less than 3%, then only two TLC plates may be used to determine the calibration coefficients.

A comparison of the results obtained for the LPS preparation by our method and the sodium borohydride–2-thiobarbituric acid procedure[10] showed good agreement within the limits of the above-mentioned error. The LPS preparation examined contained 13.5 and 1.67% of KDO and 3,6-DDH, respectively.

Thus, as far as anticipated errors are concerned, the method suggested here is comparable with spectrophotometric techniques, but is superior in respect of sensitivity and specificity.

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