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Note

Separation of tetrazolium derivatives by reversed-phase high-performance liquid chromatography

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Ditetrazolium salts are widely used for bio- and histochemical and for analytical purposes. The ditetrazolium salts are often heavily contaminated with the corresponding monotetrazolium salts which, precludes the reproducible use of the product. Column liquid[1,2], paper[3,4] and thin-layer[5–7] chromatographic methods have been developed for the analysis of various ditetrazolium derivatives. A rapid, flexible and sensitive chromatographic method was desired for the routine analysis of various ditetrazolium salts of widely varying polarity. In view of their ionic nature and hydrophobic structural elements, a reversed-phase high-performance liquid chromatographic (HPLC) method was ultimately developed using an aqueous tetrahydrofuran eluent containing lauryl sulphate counter ions. The tetrazolium derivatives studied here are listed in Table 1.

EXPERIMENTAL

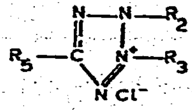
Reagent-grade mono- and ditetrazolium salts were obtained from Reanal (Budapest, Hungary) (I — IX) and Fluka (Buchs, Switzerland) (III, VIII). Reagent-grade methanol, acetonitrile, tetrahydrofuran and sodium lauryl sulphate were obtained from Reanal. Deionized water was further treated by percolating it through a bed of Soxhlet-extracted XAD-2 resin (Röhm and Haas, Philadelphia, PA, U.S.A.).

A Varian LC 5030 liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with a 10-μl loop injector and 254-nm constant-wavelength UV detector was used. Separations were carried out on a 25 × 0.4 cm I.D. stainless-steel column (Knauer, Oberursel, G.F.R.) packed with 10-μm RP-18 material (Merck, Darmstadt, G.F.R.). Solvents were filtered through GF/F glass-fibre filters (Whatman, Clifton, NJ, U.S.A.) and vacuum degassed in an ultrasonic bath. The column was thermostated at 30°C.

TABLE I

STRUCTURES OF TETRAZOLIUM DERIVATIVES

Monotetrazolium salts:



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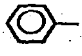
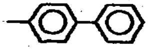
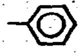
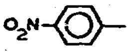
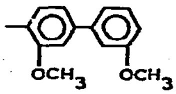
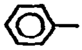
I			
II			

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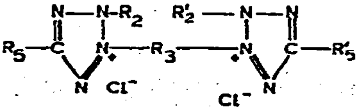
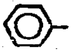
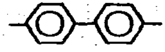
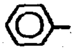
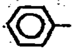
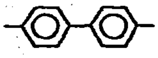
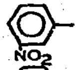
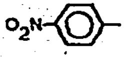
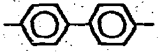
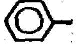
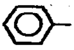
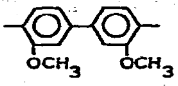
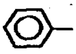
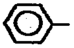
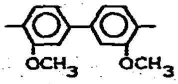
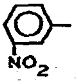
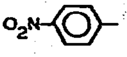
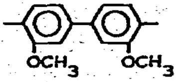
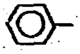
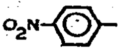
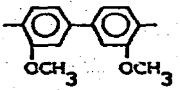
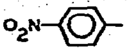


No.	R_2, R'_2	R_3	R_5, R'_5
III			
IV			
V			
VI			
VII			
VIII			
IX			

RESULTS AND DISCUSSION

Reproducible separations could not be obtained with aqueous methanol and acetonitrile eluents. Most of the components were eluted before or at the dead volume of the column. When 0.001 M sodium lauryl sulphate was added to these eluents, compounds III and VII could not be eluted at all, and the retentions of the other ditetrazolium derivatives were also excessive. Therefore, aqueous tetrahydrofuran (THF) eluents containing 0.001 M sodium lauryl sulphate were tried. The water content of the eluent was changed in 5% (v/v) steps. Satisfactory retentions were obtained with THF–water (84.5:15.5) containing 0.001 M sodium lauryl sulphate as eluent. At levels above 0.15 μg per component the retention volume of the ditetrazolium derivatives was dependent on the amount of the derivative injected. The k' values determined by injecting about 75 ng of each are listed in Table II.

As expected, the monotetrazolium derivatives were eluted before the ditetrazolium derivatives. Among the ditetrazolium derivatives there are two main groups: those without and those with methoxy substituents. Within each group there is a

TABLE II

k' VALUES OF THE TETRAZOLIUM DERIVATIVES DETERMINED ON RP-18 REVERSED-PHASE PACKING AT 30°C USING TETRAHYDROFURAN–WATER (84.5:15.5) CONTAINING 10^{-3} M SODIUM LAURYL SULPHATE AS ELUENT

No.	*k'*	*Type*	*No.*	*k'*	*Type*
I	3.8	Mono	VI	20	Dimethoxy
II	3.3	Mono	VII	9.4	m-NO_2-dimethoxy
III	15	Parent compound	VIII	9.0	p-NO_2-dimethoxy
IV	9.1	m-NO_2	IX	4.9	Di-NO_2-dimethoxy
V	8.7	p-NO_2			

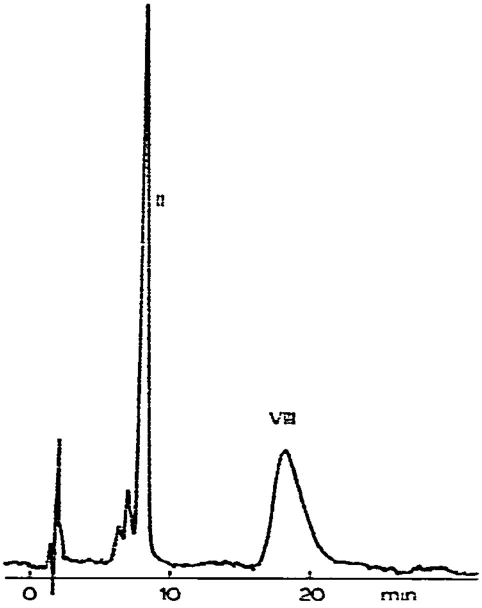


Fig. 1. Separation of monotetrazolium derivative II (a contaminant) and ditetrazolium derivative VIII (main product) on a Knauer 0.4 × 25 cm I.D. RP-18 column with THF–water (84.5:15.5) containing 0.001 M sodium lauryl sulphate as eluent.

parent compound (III and VI), a *m*-nitro-substituted (IV and VII) and a *p*-nitro-substituted (V and VIII) derivative. Compound IX has no "pair". It can be seen that addition of the two methoxy groups always increases the *k'* value (*cf.*, III *vs.* VI, IV *vs.* VII and V *vs.* VIII). Within the main groups the addition of a nitro group decreases *k'*, and the extent of the decrease depends on the position of the nitro group. The elution order is the same in both groups: V, IV, III and VIII, VII, VI. The above conclusions could be used to predict the approximate retention order of other, similarily substituted tetrazolium derivatives.

This system was used to obtain a general idea of the purity of experimental batches of various ditetrazolium derivatives. The chromatogram of VIII which contains II as impurity is shown in Fig. 1.

The concentration of I in pilot-plant charges of III and that of II in VIII were determined with a slightly different eluent: THF–water (82.5:17.5) containing 10^{-3} *M* sodium lauryl sulphate. This eluent gave a better separation and quantitation of the various monotetrazolium derivatives. Owing to the excessive retention volumes of the corresponding ditetrazolium derivatives, a minimum of three injections could be made before III or VIII of the first injection started to elute.

The method has been used successfully for quality control purposes to aid the production of III and VIII.

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