CHROM. 6199

Detection of catecholamines and related compounds with diazonium reagents by thin-layer chromatography

The quantitative assay procedures used in clinical chemistry laboratories for catecholamines and related alkyl- and arylamines and acids in biological fluids are lengthy and require special equipment and techniques[1,2]. Several screening procedures including paper chromatographic[3-5], spot test[6-8] and thin-layer chromatographic (TLC) techniques[9-13] have been evaluated. Many of these methods are time-consuming and cumbersome, while others are non-specific or not sufficiently comprehensive. In this study, a new TLC technique was used[14] to separate the compounds in question. Various diazonium salt solutions were evaluated as reagents for making spots visible. A common reaction of diazonium compounds is coupling with phenols or amines to form highly colored azo compounds. Phenols couple fastest under mildly basic conditions, and the chromatograms were therefore exposed to ammonia vapor or, to form more stable colors, they were sprayed with 10% sodium carbonate solution. Because of the subjectiveness of determining colors, these have been omitted from the tables; they range from light yellow to dark purple.

Materials and methods

Separations were accomplished with the Chromat/O/Screen Analysis Kit for Catecholamines (Eastman Kodak No. 13123). The sorbent layer is cellulose and the solvent system is a chromatography gel composed of butanol–ethanol–ammonium hydroxide–water (10:1:2:4). Diazonium salts were purchased from Eastman Organic Chemicals, Harleco, and Calbiochem Company. Table I shows the structure of the various salts tested.

Compounds used as standards were purchased from Sigma Chemical Company, Nutritional Biochemicals Corporation (NBC), K & K Laboratories Inc., Mann Research Laboratories and Eastman Organic Chemicals. Standard solutions were prepared at a concentration of 5 μg/ml. These solutions were serially diluted and chromatographed. Tables II–V include the R_F value and detection limit of each compound with each diazonium reagent.

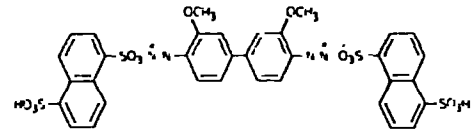
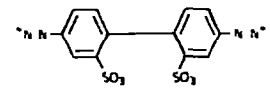
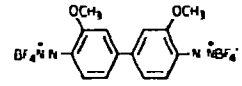
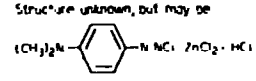
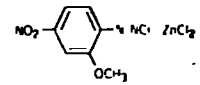
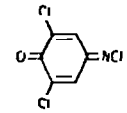
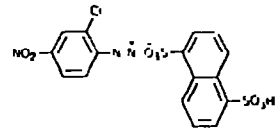
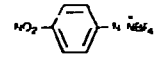
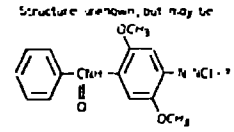
Discussion

Diazo coupling is one of a wide variety of electrophilic aromatic substitution reactions. Strongly activating substituents (electron-releasing) such as $-NR_2$ should cause the substrate to react faster than moderately activating groups such as $-OCH_3$, $-NHCO$ and $-C_6H_5$. Substrates containing deactivating (electron-withdrawing) substituents, such as $-NO_2$, $-SO_3H$ and $-Cl$, should react the slowest. If the substituent, on the other hand, is substituted on the attacking electrophile, *i.e.*, the diazonium salt, the situation is reversed. Electron-withdrawing groups increase the concentration of the positive charge and hence the electrophilicity of the ArN_2^+. Salts of the diazonium compounds, such as tetrafluoroborate, tri- or tetrachlorozincate and sulfonate, in aqueous solutions do not alter the reaction; they are more stable to light and oxidation–reduction decomposition than the corresponding free base.

TABLE I

DIAZONIUM SALTS

All salts were freshly prepared (< 1 h old). Solutions were made at a concentration of 0.1% in water.

No.	Compound	Molecular formula	Structural formula
1	3,3′-Dimethoxy-4,4′-biphenyltetrazonium bis(1,5-naphthalenedisulfonate)	$C_{34}H_{26}N_4O_{14}S_4$	
2	4,4′-Biphenyltetrazonium-2,2-disulfonate	$C_{12}H_6N_4O_6S_2$	
3	3,3′-Dimethoxy-4,4′-biphenyltetrazonium bis(tetrafluoroborate)	$C_{14}H_{12}B_2F_8N_4O_2$	
4	*p*-Dimethylaminobenzenediazonium tetrachlorozincate	$C_8H_{11}Cl_4N_3Zn$	
5	2-Methoxy-4-nitrobenzenediazonium trichlorozincate (Fast Red B)	$C_7H_6Cl_3N_3OZn$	
6	N-2,6-Trichloro-*p*-benzoquinoneimine[a]	$C_6H_2Cl_3NO$	
7	2-Chloro-4-nitrobenzenediazonium-1,5-naphthalenedisulfonate	$C_{16}H_{10}ClN_3O_8S_2$	
8	*p*-Nitrobenzenediazonium tetrafluoroborate	$C_6H_4BF_4N_3O_2$	
9	4-Benzamido-2,5-dimethoxybenzenediazonium chloride (Fast Blue Salt RR)	—	

[a] Not a diazonium structure but contains an active nitrogen linkage. This compound was made 0.1% in 70% ethanol.

TABLE II

R_F VALUES AND DETECTION LIMITS OF CATECHOLAMINES AND RELATED COMPOUNDS

Compound	*Abbreviation*	$R_F \times 100$	*Detection limits of diazonium salts (μg)*								
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Metanephrine	MN	94	1.0	1.0	0.5	—[a]	0.2	0.2	1.0	0.03	1.0
Normetanephrine	NMN	75	0.2	1.0	0.1	—	0.1	0.2	0.5	0.01	0.4
3,4-Dihydroxyphenylethylamine	DOPAMINE	48	2.0	2.0	1.0	2.0	1.0	1.0	0.5	0.03	2.0
Epinephrine	E	40	2.0	1.0	2.0	—	2.0	5.0	5.0	0.05	1.0
4-Hydroxy-3-methoxyphenylacetic acid	HVA	40	0.5	0.5	0.5	—	0.2	—	0.2	0.01	5.0
Norepinephrine	NE	32	1.0	1.0	0.5	2.0	1.0	1.0	0.5	0.02	0.4
4-Hydroxy-3-methoxymandelic acid	VMA	25	0.1	0.2	0.1	—	0.1	0.2	0.1	0.01	0.4
3,4-Dihydroxyphenylalanine	DOPA	7	2.0	2.0	2.0	—	—	2.0	2.0	0.05	4.0

[a] No reaction at $\leqslant 5$ μg of compound.

TABLE III

R_F VALUES AND DETECTION LIMITS OF PHENOLIC ACIDS

Compound	*Abbreviation*	$R_F \times 100$	*Detection limits of diazonium salts (μg)*								
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
o-Hydroxyphenylacetic acid	*o*-HPAA	60	0.2	0.2	0.1	—[a]	0.1	0.1	0.1	0.5	0.4
p-Hydroxyphenyllactic acid	*p*-HPLA	44	2.0	1.0	1.0	—	1.0	—	0.2	0.3	—
p-Hydroxyphenylacetic acid	*p*-HPAA	38	1.0	0.5	0.5	—	0.5	—	0.2	0.1	—
m-Hydroxybenzoic acid	*m*-HBA	37	0.5	0.5	0.5	—	0.5	0.5	0.2	0.5	—
p-Hydroxymandelic acid	*p*-HMA	20	1.0	0.5	0.5	—	0.1	3.0	0.1	0.1	4.0
Vanillic acid	VA	14	0.2	0.2	0.2	—	0.1	1.0	0.1	0.01	1.0
3,4-Dihydroxyphenylacetic acid	di-HPAA	10	2.0	2.0	2.0	2.0	2.0	2.0	2.0	NA[b]	0.4

[a] No reaction at $\leqslant 5$ μg of compound.
[b] NA = not available.

TABLE IV

R_F VALUES AND DETECTION LIMITS OF INDOLES AND IMIDAZOLES

Compound	*Abbreviation*	$R_F \times 100$	*Detection limits of diazonium salts (μg)*								
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Tryptamine	Ta	96	0.2	3.0	0.2	—[a]	—	1.0	—	2.0	3.0
5-Hydroxytryptamine (Serotonin)		80	0.2	1.0	0.5	2.0	0.2	0.5	0.2	0.4	0.2
Imidazole	I	80	3.0	2.0	2.0	—	—	—	2.0	0.5	—
5-Hydroxyindoleacetic acid	5-HIAA	39	0.1	0.5	0.1	0.5	0.1	0.1	0.1	0.5	0.1
Tryptophan	To	38	0.3	0.5	0.3	—	1.0	3.0	—	0.4	0.5
5-Hydroxytryptophan	5-HTo	35	0.1	0.5	0.1	0.2	0.1	0.2	0.1	NA[b]	0.1
Imidazolelactic acid	ILA	28	0.2	0.2	0.2	—	0.1	—	2.0	0.1	5.0
Imidazoleacetic acid	IAA	26	0.5	0.1	0.5	—	0.1	—	0.2	0.5	5.0

[a] No reaction at $\leqslant 5$ μg of compound.
[b] NA = not available.

TABLE V

R_F VALUES AND DETECTION LIMITS OF AMINES AND PHENOLIC COMPOUNDS

Compound	$R_F \times 100$	*Detection limits of diazonium salts (μg)*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Phenol	98	—[a]	—	—	—	1.0	1.0	1.0	—	—
Tyramine	94	0.3	1.0	0.3	—	4.0	1.0	—	0.4	—
Orcinol	92	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.1
Sympatol	90	2.0	2.0	1.0	—	0.1	3.0	0.2	0.3	4.0
Histamine	82	0.3	0.3	0.3	—	1.0	0.3	—	4.0	4.0
Ethanolamine	54	2.0	2.0	0.5	—	—	1.0	4.0	1.0	—
Tyrosine	34	2.0	1.0	1.0	—	1.0	—	0.1	0.5	3.0
Alanine	30	5.0	2.0	1.0	—	—	—	1.0	0.5	—
Asparagine	10	—	—	3.0	—	—	1.0	—	2.0	—
Glutamine	10	—	—	3.0	—	—	1.0	—	4.0	—

[a] No reaction at $\leqslant 5$ μg of compound.

Our results support the electronic considerations discussed above. Thus, Reagent 8, with an $-NO_2$ group in the *para* position, has the best overall sensitivity, whereas Reagent 4, containing a $-N(CH_3)_2$ group, is the least active. Reagent 4 reacts only with dihydroxy aromatic compounds or monohydroxy aromatic nuclei activated by other groups.

From the results, it can be seen that substitution on the aromatic nucleus is important in choosing sensitive reagents for making spots visible in TLC. On the other hand, because of the variables inherent in TLC (temperature, moisture, pH of adsorbent, etc.), empirical results must be collected, despite theoretical considerations, before a good choice of reagent can be made.

We conclude that *p*-nitrobenzenediazonium tetrafluoroborate (compound 8, Tables I–V) is the best reagent of those tested for making spots of biologically important metabolites (VMA, HVA, E and NE) visible in TLC.

We are grateful to ALBERT BAITSHOLTS of Eastman Kodak Company for supplying some diazonium compounds and to THANIA ESPINOSA and OFELIA BECERRA for technical assistance.

TLC Corporation,
Miami, Fla. 33156 (U.S.A.)

R. ROSER
P. M. TOCCI

1 C. SOBEL AND R. J. HENRY, *Amer. J. Clin. Pathol.*, 27 (1957) 923.
2 L. E. THOMAS, *Amer. J. Clin. Pathol.*, 28 (1957) 605.
3 L. H. LASSBERG AND S. SHIMOSATO, *J. Appl. Physiol.*, 21 (1966) 1929.
4 E. G. MCGREER AND W. H. CLARK, *J. Chromatogr.*, 14 (1964) 107.
5 G. L. MATTOCK, *J. Chromatogr.*, 16 (1964) 254.
6 R. ROBINSON, J. RATCLIFFE AND P. SMITH, *Clin. Pathol.*, 12 (1959) 541.
7 S. E. GITLOW, L. ORNSTEIN, M. MENDLOWITZ, S. KHASSIS AND E. KRUK, *Amer. J. Med.*, 8 (1960) 921.
8 L. E. ROGERS AND F. S. PORTER, *Proc. South Loc. Pediatr. Res.*, Abstr. 110 (Nov. 1968).
9 F. H. SCHNEIDER AND C. N. GILLIS, *Biochem. Pharmacol.*, 14 (1965) 623.
10 W. P. DEPOTTER, R. F. VOCHTEM AND A. F. DESCHAEPDRYVER, *Experientia*, 21 (1965) 482.
11 A. VAHIDI AND D. V. S. SANKAR, *J. Chromatogr.*, 43 (1969) 135.
12 J. M. C. GUTTERIDGE, *Clin. Chim. Acta*, 21 (1968) 211.
13 A. BJORKLUND, B. FALCH AND R. HAKANSON, *Anal. Biochem.*, 35 (1970) 264.
14 P. M. TOCCI, *Symp. Int. Chromatogr. Électrophorèse, VI, Bruxelles, 1971*, Presses Académiques Européennes, Brussels.

Received May 2nd, 1972