Diazotization of Catecholamines and Their Analogs and Metabolites for Urinary Screening Tests: Chemical Aspects

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Coupling of diazotized p-nitroaniline to catecholamines and their metabolites in urine has been proposed for use in screening for secreting neuroblastoma in childhood. We have coupled diazotized p-nitroaniline to catecholamines, derivatives, and metabolites and examined the reaction products by thin-layer chromatography and physico-chemical methods (ultraviolet spectra, mass spectrometry, nuclear magnetic resonance). We conclude that during diazotization, products containing a p-hydroxybenzyl alcohol or a p-hydroxybenzoic acid structure (e.g., vanillic acid, vanilmandelic acid, 3-methoxy-4-hydroxyphenylethylenglycol, metanephrine, normetanephrine, synephrine, and isoproterenol) react with a diazonium cation, with release of their alcohol or acid moiety. Therefore the mentioned qualitative screening methods are very nonspecific. In contrast, thin-layer chromatographic screening methods provide complete separation and unambiguous identification of these metabolites and are to be preferred for use in detecting secreting neuroblastoma in childhood.

Additional Keyphrases: screening for secreting neuroblastoma • F, data (thin-layer chromatography) • UV, NMR data • urinary phenolic acids and azo-derivatives

Children suffering from secreting neuroblastoma may excrete particularly high amounts of DA, HVA, iso-HVA (the 4-O-methylated counterpart of HVA), NE, VMA, and MHPG in their urine. In some cases, excretion of dopa and its catabolite VLA is also observed. The occurrence of abnormally high amounts of VMA in urine is often used as a diagnostic tool for this disease (1–3).

Several rapid test-tube and spot-test screening methods (4–6) have been described for semiquantitative determination of VMA in urine. In one colorimetric procedure, VMA is coupled to diazotized aromatic amines such as p-nitroaniline, to produce a violet azo dye.

It was empirically established that in most patients with secreting neuroblastoma, treatment of urine with diazotized p-nitroaniline resulted in a predominant violet color, and this has been attributed solely to the presence of increased amounts of VMA.

However, one must be aware that human urine contains more than 30 phenolic acids, phenolic alcohols, and phenolamines, which produce various colors on such treatment, and a recent report has shown that most compounds that possess a guaiacol structure (such as guaiacol, VA, VMA, MHPG, vanillyl alcohol, metanephrine, and normetanephrine) produce with diazotized p-nitroaniline this same “characteristic” violet color (7), a finding that implies the nonspecificity of the violet color in cases of secreting neuroblastoma. DA, dopa, VLA, and HVA do not contribute to this color, because their azo derivatives are respectively pink, pale green, and greyish blue for the two last compounds. Iso-HVA, the excretion of which is relatively more important in cases of secreting neuroblastoma than in normal children, produces a deep-purple azo derivative, and also contributes to the “violet” color (8).

Despite increasing evidence that the VA that appears in urine in cases of secreting neuroblastoma is endogenous (9), some of it may also be exogenous, because it can derive from vanilla flavoring used in food. Moreover, several guaiacol derivatives are excreted after administration of certain medications (6, 10). Thus the diet of the patient is a very important factor in avoiding falsely positive results. When the urine is to be treated with diazotized p-nitroaniline, patients must have been on a special diet that is devoid of aromatic compounds for at least one day.

To compare the efficiency of the different screening tests for the detection of VMA in urine, we studied the chemical basis of its diazotization. Our results indicate that compounds possessing either a p-hydroxybenzyl

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alcohol structure (VMA, MHPG, metanephrine, nor-
metanephrine, SY, and IP) or a p-hydroxybenzoic acid
structure (VA) react with diazotized p-nitroaniline with
release of the alcohol or acid moiety. This reaction oc-
curs specifically at an alkaline pH, whereas at acid pH
other diazonium derivatives are also formed. An im-
portant consequence of this mechanism is that VA,
VMA, MHPG, normetanephrine, and metanephrine,
when diazotized at the usual alkaline pH, give rise to the
same violet end-product, namely, 4-PNBD-guaiacol.
Although the presence of 4-PNBD-guaiacol can be de-
termined by thin-layer chromatography, its occurrence
provides no information about the exact composition of
methylated catabolites of NE in urine, and therefore
about the presence of increased concentrations of
VMA.

Materials

p-Nitrobenzylidiazonium fluoroborate and p-nitro-
aniline hydrochloride were obtained from ICN-K&K
Laboratories, Plainview, N.Y. 11803. D,L-Isoproterenol
and D,L-synephrine were from Aldrich Europe, B-2340
Beerse, Belgium. Pyrocatechol was purchased from
Fluka AG, CH-9470 Buchs, Switzerland. Iso-HVA was
kindly supplied by F. Hoffmann-La Roche & Co., 4000
Basel, Switzerland. All other reference products were
purchased from Sigma Chemical Co., St. Louis, Mo.
63178.
The chemicals were of the purest grade available.
Pre-coated 20 × 20 cm silica gel and cellulose plates
for thin-layer chromatography were from Merck, 6100
Darmstadt, Germany (without fluorescence indica-
ator).

Methods

Synthesis of Azo Derivatives

Several azo derivatives, depicted in Figure 1, were
prepared as follows:

4-PNBD-phenol: Stir mixture of equimolar amounts
of SY (445 mg) and PNBD fluoroborate, dissolved in
150 ml of phosphate buffer (0.1 mol/liter, pH 6), at 4 °C
in the dark under a nitrogen atmosphere for 24 h. Dis-
card the brown precipitate isolated by filtration through
a sintered-glass filter. Add ethanol to the filtrate to
 crystallize the 4-PNBD-phenol present in this mixture.
The product is obtained as brown slivers [yield, 155 mg
(24%)] after lyophilization.

3-PNBD-synephrine: Add a solution of PNBD hy-
drochloride (obtained by stirring a mixture of 2.5 g of
p-nitroaniline hydrochloride and 1.5 g of sodium nitrite
in 120 ml of 1 mol/liter hydrochloric acid for 10 min at
0 °C, and then increasing the pH to 3 with 3 mol/liter
sodium hydroxide) to a solution of 2 g of SY in 100 ml
of an equimolar mixture of ethanol and acetate buffer
(0.1 mol/liter, pH 3). Stir the final mixture at 4 °C in the
dark for 24 h, then pur and pour it into ice-water. Isolate
the resulting yellow precipitate on a sintered-glass filter.
After recrystallizing the precipitate twice from ethanol/benzene, one obtains 3-PNBD-synephrine [brown
needles; yield, 49 mg (1.3%)].

6-PNBD-phenylephrine: Stir a mixture of equimolar
amounts of PHE (400 mg) and PNBD fluoroborate,
dissolved in phosphate buffer (0.1 mol/liter, pH 6), at
4 °C in the dark under a nitrogen atmosphere for 7 h.
Collect the orange precipitate on a sintered-glass filter,
and recrystallize it from methanol/ethanol [yellow
needles; yield, 390 mg (52%)].

4-PNBD-catechol: Dissolve a mixture of equimolar
amounts of pyrocatechol (240 mg) and PNBD fluoro-
borate in 75 ml of acetate buffer (0.1 mol/liter, pH 4)
and stir in the dark at 4 °C for 20 h. Extract the sus-
pension with ethyl acetate and dry the organic phase
over anhydrous magnesium sulfate. Add heptane to the
remaining ethyl acetate solution and crystallize 4-
PNBD-catechol present in this mixture [brown powder;
yield, 130 mg (50%)].

4-PNBD-guaiacol: Add a solution of PNBD hydro-
chloride (obtained by stirring a mixture of 350 mg of
p-nitroaniline hydrochloride and 200 mg of sodium
nitrite in 20 ml of 2 mol/liter hydrochloric acid for 10
min at 0 °C and then increasing the pH to 8 with 1
mol/liter sodium hydroxide) to a solution of 328 mg of
VA in 40 ml of carbonate buffer (0.1 mol/liter, pH 8).
Stir the final mixture for 30 min at 4 °C in the dark.
Extract the resulting suspension with ethyl acetate and
dry the organic phase with anhydrous magnesium sul-
fate. Evaporate the solvent under suction, and recrys-
tallize the residue twice from ethanol/water [red-brown
slivers; yield, 138 mg (26%)].

Analysis of Azo Derivatives

Physical methods. Infrared spectra of the solids
dispersed in KBr were obtained with a Unicam SP 1000
spectrophotometer (Pye Unicam Ltd, Cambridge,
England). Mass spectra were taken with a A.E.I. MS 902
S mass spectrometer (A.E.I., M31 2LD Manchester, U.
K.) at 15 eV ionizing energy. Ultraviolet measurements
were made on a Cary 118 spectrophotometer (Cary In-
struments, Monrovia, Calif. 91016). Nuclear magnetic
resonance spectra were recorded at 270 MHz with a
Bruker Spectrisp HPDX 270 spectrometer (Bruker
Physik, 7501 Karlsruhe, Forchheim, Germany), with use
of solutions in deuterio-trifluoroacetic acid. Signal pos-
tions are reported in ppm downfield to tetramethyl
silane. Melting points were determined under reduced
pressure in sealed capillary tubes in a “Tottoli” melting
point apparatus (W. Büchi, CH-9230 Fawil, Swit-
zerland).

Ascending thin-layer chromatography; pH-depen-
dence of azo substitution. Stir a mixture of equimolar
amounts of PNBD fluoroborate and either SY, PHE,
IP, DA, VA, VMA, MHPG, normetanephrine, or
metanephrine (0.1 mol/liter), dissolved in 10 ml of
buffers with increasing pH values (pH 2–5: 0.1 mol/liter
acetate buffer; pH 6–8: phosphate buffer 0.1 mol/liter;
and pH 9: 0.1 mol/liter carbonate buffer). Allow reac-
tions to proceed in the dark at 4 °C until completion
(one to four days).

Dilute the reaction mixtures with 10 ml of ethanol (for
SY); or 10 ml of methanol (for PHE and DA). Lyophilize the reaction mixtures and extract the azo derivatives from the buffer with 20 ml of ethanol (for IP) or 20 ml of methanol (for VA, VMA, MHPG, and metanephrine). Centrifuge these alcoholic extracts at low speed (1000 \times g, 5 min) to precipitate suspended salts.

Spot identical volumes (10–50 \mu l) of the alcohol or alcohol/water solution of a single compound diazotized at different pH values on the same thin-layer chromatographic plate. Spot azo derivatives of SY, PHE, IP, and DA on silica gel plates, and elute the compounds with ethyl acetate/formic acid/water (17/2/1 by vol; System I). Spot azo derivatives of VA, VMA, MHPG, and metanephrine either on (a) cellulose plates, and elute with ethanol/conc ammonium/sodium carbonate (0.1 mol/liter), (1/1/8 by vol; System II), or with isopropyl alcohol/ammonia/sodium carbonate (0.1 mol/liter) (5/1/4 by vol; System III), or (b) silica gel plates, and elute with benzene/ether (7/3 by vol; System IV).

The (ascending) chromatographic separations are performed at 20–22 °C under an atmosphere saturated with the respective elution mixture.

**Results**

The reactions involved are depicted in Figure 1.

**Diazotization of Catecholamine Analogs (Phenol Derivatives)**

We first consider the diazotization reaction of the catecholamine analogs (SY and PHE) under different pH conditions. With regard to the aromatic ring, these compounds are the simplest of the considered structures—a substituted phenyl ring, as compared to a substituted catechol ring for catecholamines and a substituted guaiacol ring for the 3-O-methylated catecholamine metabolites. PNBD-fluoroborate is used because it is soluble in all buffers tested without notable decomposition, and thus allows for a well-controlled pH of the diazotization reaction.

Mixtures of equimolar amounts of drug and

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**Fig. 1. Diazotization of catecholamines, analogs, and metabolites with PNBD fluoroborate**

Azo derivatives are formed in acid (A) and (or) alkaline (B) medium. The position of the azo substitution at the aromatic ring is derived from the native compound (when the side-chain remains) or from phenol, catechol, and guaiacol (when the side-chain is cleaved off).
PNBD-fluoroborate, dissolved in buffer of pH 2, 3, . . . 9, are stirred at 4 °C in the dark. After completion, reaction specificity is first determined by examining the end-products by means of thin-layer chromatography, as described above and in Table 1. Diazotization of PHE leads to only one end-product at all pH values examined. In contrast, SY gives rise to two end-products, one of which is present at all pH values while the other is only observed after diazotization at acidic pH (Table 1). In a subsequent step, the azo derivatives of SY and PHE are synthetized as described in the experimental procedure. The physicochemical characteristics of these derivatives (Table 2) indicate that, whereas diazotization of PHE gives rise to 6-PNBD-phenylephrine, diazotization of SY leads to the formation of 3-PNBD-synephrine at acid pH and to 4-PNBD-phenol at acid and alkaline pH. This last compound contains no longer an ethanolamine side-chain and therefore migrates very fast in thin-layer chromatographic system I. As determined by thin-layer chromatography and subsequent ultraviolet spectroscopy, the maximum yield of 6-PNBD-phenylephrine is 92%, at pH 6. The maximum yield of 3-PNBD-synephrine (5%) is at pH 3 and that of 4-PNBD-phenol (50%) at pH 8 (Figure 2).

Diazotization of Catecholamines (Catechol Derivatives)

The diazotization of IP at various pH's results in the formation of several catechol and noncatechol substances, as indicated by thin-layer chromatography (Table 1 and Figure 3). At pH 7 or above, one of the catechol-containing azo derivatives migrates fast (RF, 0.86), indicating that the ethanolamine side chain of IP might have been lost. Indeed, this spot may be attributed to 4-PNBD-catechol, because its migration and its color after staining with either ferric chloride or ammonia is identical to that of 4-PNBD-catechol, independently prepared by diazotizing pyrocatechol (see the experimental procedure and Table 2). For similar reasons, the catechol-containing azo derivative, which migrates not far beyond native IP (RF 0.46 vs. RF 0.28 for IP), is probably 6-PNBD-isoproterenol (Table 1). Thus, when diazotizing SY and IP at weak alkaline pH, substantial amounts of, respectively, 4-PNBD-phenol and 4-PNBD-catechol are obtained. These results are consistent with the fact that compounds possessing a p-hydroxybenzyl alcohol structure may be attacked by the PNBD-cation with removal of the alcoholic side chain (Figure 1). As control, we investigated diazotiza-

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**Table 1. Thin-layer Chromatographic Data**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(Solvent system) and RF value</th>
<th>Native color</th>
<th>Color after staining with</th>
<th>Max. yield at pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>**A. For Native and Diazo-</td>
<td></td>
<td></td>
<td>NH₃</td>
<td>Fe₃Cl₃</td>
</tr>
<tr>
<td>phenethylamine and Derivatives**</td>
<td></td>
<td></td>
<td>Fe₃Cl₃</td>
<td>Fe₃Cl₃</td>
</tr>
<tr>
<td>Phenylephrine (PHE)</td>
<td>(I) 0.21</td>
<td>n.c.</td>
<td>n.c.</td>
<td>n.c.</td>
</tr>
<tr>
<td>6-PNBD-phenylephrine</td>
<td>(I) 0.44</td>
<td>orange</td>
<td>red</td>
<td>n.c.</td>
</tr>
<tr>
<td>Synephrine (SY)</td>
<td>(I) 0.20</td>
<td>n.c.</td>
<td>n.c.</td>
<td>n.c.</td>
</tr>
<tr>
<td>4-PNBD-phenol</td>
<td>(I) 0.95</td>
<td>orange</td>
<td>red</td>
<td>n.c.</td>
</tr>
<tr>
<td>3-PNBD-synephrine</td>
<td>(I) 0.30</td>
<td>orange</td>
<td>red</td>
<td>n.c.</td>
</tr>
<tr>
<td>Isoproterenol (IP)</td>
<td>(I) 0.28</td>
<td>n.c.</td>
<td>brown</td>
<td>purple</td>
</tr>
<tr>
<td>4-PNBD-catechol</td>
<td>(I) 0.86</td>
<td>orange</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>6-PNBD-isoproterenol</td>
<td>(I) 0.46</td>
<td>orange</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>Dopamine (DA)</td>
<td>(I) 0.28</td>
<td>n.c.</td>
<td>brown</td>
<td>purple</td>
</tr>
<tr>
<td>6-PNBD-dopamine</td>
<td>(I) 0.52</td>
<td>orange</td>
<td>purple</td>
<td>purple</td>
</tr>
</tbody>
</table>

**B. For Diazo-| phenethylamine and Derivatives** |                               | Obtained on diazotization of | | |
| phenethylamine and Derivatives** |                               | Solvent system and RF value | II | III | IV | Color |
| 4-PNBD-guaicarol          | VA (A, B)                     | 0.32          | 0.97        | 0.62        | violet         |
|                          | VMA (A, B)                    | 0.32          | 0.97        | 0.62        | violet         |
|                          | MHPG (A, B)                   | 0.32          | 0.97        | 0.62        | violet         |
|                          | Metanephrine (A, B)           | 0.32          | 0.97        | 0.62        | violet         |
|                          | Normetanephrine (A, B)        | 0.32          | 0.97        | 0.62        | violet         |
| 5-PNBD-VMA                | VMA (A)                       | 0.47          | 0.00        | 0.00        | greyish blue   |
| 5-PNBD-MHPG               | MHPG (A)                      | 0.37          | 0.00        | 0.00        | greyish blue   |
| 5-PNBD-metanephrine       | Metanephrine (A)              | 0.47          | 0.00        | 0.00        | greyish blue   |
| 5-PNBD-normetanephrine    | Normetanephrine (A)           | 0.47          | 0.00        | 0.00        | greyish blue   |

* n.c.: no color observed. Native catecholamines are colorless but may be oxidized with an aqueous CrO₃-spray. Azo derivatives are orange and change their color on exposure to NH₃-vapors. Subsequent spraying with aqueous Fe₃Cl₃ and aqueous sodium bicarbonate makes visible the catechol ring (15). The RF value denotes the migration of a compound as compared to the migration of the solvent front (RF = 1). This RF value is low for substances with, and high for substances without, an ethanolamine side-chain.

° See also Figure 2.

Azo derivatives are formed in acid (A) or alkaline pH (B), or both.
Table 2. Physical-chemical Data on Diazotized Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>NMR-spectrum: aromatics</th>
<th>UV-spectrum</th>
<th>Melting point, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-PNBD-phenylephrine</td>
<td>PNBD-molety 8.56</td>
<td>390</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td>Substituted hormone</td>
<td>log</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.14 8.35 7.63 7.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-PNBD-phenol</td>
<td>d,2 d,2 d,1 s,1 d,1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-PNBD-synephrine</td>
<td>d,2 d,2 d,2 d,2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-PNBD-catechol</td>
<td>d,2 d,2 s,1 d,1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-PNBD-guaiacol</td>
<td>d,2 d,2 d,1 s,1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Nuclear magnetic resonance spectrum (NMR-spectrum): For each aromatic signal is given the delta value in ppm, multiplicity (s = singlet, d = doublet with a J value of ±9 Hz), and number of protons. The aliphatic signals of the ethanolamine side-chain are only present in the spectrum of 6-PNBD-phenylephrine and 3-PNBD-syephrine, and are similar to those of native synephrine (data not shown).

The ultraviolet-spectrum of 3-PNBD-syephrine contains two maxima, which are typical for azosubstitution next to the aromatic hydroxy group (16). The spectrum of the azo derivatives is recorded with them in ethanolic solution at 0.1 mmol/liter concentration.

The mass spectrum contains the original peak for 4-PNBD-phenol (m/e = 243), 4-PNBD-catechol (m/e = 259), and 4-PNBD-guaiacol (m/e = 273). Those data, along with those obtained from infrared-spectroscopy, agree with the structures for the azo derivatives proposed in Figure 1.

Fig. 2. Diazotization of synephrine and phenylephrine: yield of azo derivatives as function of the pH

After diazotization at different pH values, identical volumes of the reaction mixture of SY (or PHE) are applied on the same thin-layer chromatographic plate. After migration (System I), spots are scraped off the plate, and azo derivatives are extracted from the gel with methanol/HCl (99/1 by vol). The alcoholic extracts are centrifuged at low speed to precipitate suspended salts. A_{max}-values obtained by ultraviolet-chromatography of the extracts are compared with the corresponding calibration curves (obtained by similar treatment of increasing amounts of the synthetized azo derivatives) to obtain yield of the azo derivatives as a function of the pH. Yield of 4-PNBD-phenol (middle curve), 3-PNBD-syephrine (bottom curve) and 6-PNBD-phenylephrine (top curve) are expressed in percent of the theoretical maximum (the initial amount of SY or PHE).

Fig. 3. Thin-layer chromatography of products of the reaction of isoproterenol with PNBD fluoroborate, as a function of the pH

Spots correspond to isoproterenol (IPR), or reaction products of isoproterenol with PNBD fluoroborate at pH 2 to 9, 4-PNBD catechol (PNC), and PNBD-fluoroborate. Azo derivatives are made visible after migration on the silica gel plate, with NaF-vapors. Control tests indicate that the variety of the substances is not due to the degradation of the reaction mixture on spotting or migration on the plate.

Diazotization of 3-O-Methoxylated Catecholamine Metabolites (Guaiacol Derivatives)

Next we studied the diazotization of VA, VMA, MHPG, normetanephrine, and metanephrine at pH values between 2 and 9, analyzing the end products by means of thin-layer chromatography as described in the experimental procedure (Table 1). At the higher pH values, diazotization of the five metabolites leads to one observable end product. The color and migration characteristics in three distinct chromatographic systems are such that this product appears to be the same for each of the five metabolites (Table 1). The compound is synthesized by diazotization of VA and characterized as 4-PNBD-guaiacol by physicochemical methods (experimental procedure and Table 2). These compounds possess, as for NE and its derivatives, a 2-hydroxybenzyl alcohol structure of a 2-hydroxybenzoic acid structure, and azo attack at alkaline pH
occurs at the C-1-position under release of the alcohol or carbonyl moiety. Because VA, VMA, MHPG, normetanephrine, and metanephrine not only possess different side chains (alcohol or carbonyl moieties), but also a guaiacol ring, their PNBD-derivatives are identical.

When considering the diazotization of these five metabolites at weakly acid pH, 4-PNBD-guaiacol remains the predominant end product, whereas for VMA and MHPG, an additional end product is formed as well (Table 1). We suppose this to be 5-PNBD-VA for VMA, 5-PNBD-MHPG for MHPG, 5-PNBD-metanephrine for metanephrine, and 5-PNBD-normetanephrine for normetanephrine.

**Discussion**

**Chemical Aspects**

The major kind of reactions of diazonium cations is their aromatic substitution to benzene derivatives, leading to the formation of colored azo dyes. This substitution is favored at the para position of strongly activating groups such as O\(^{-}\) (at alkaline pH) or \(-\text{OH}\) (at acid pH), whereas a methoxy group is only slightly activating (11). Thus hydroxyphenyl derivatives are diazotized at the para position with respect of the aromatic hydroxyl group. It this position is already substituted by an alkyl group, azo-coupling will proceed at the ortho position (12). However, there are exceptions.

If the para position of an aromatic hydroxyl group is substituted by a carboxyl or a CH(OH)-R group, azo coupling at alkaline pH will occur at this position, while the carboxyl or CH(OH)-R group is cleaved (12). This unusual behavior may be attributed to the electron-donating behavior of the COO\(^{-}\)- and CH(O\(^{-}\))-R groups, leading to an increased electron density at their attachment point on the ring and so favoring there the electrophilic azo addition. Thus, whereas PHE is diazotized in the para position of the aromatic hydroxyl group (according to the general rule), SY (as a p-hydroxybenzyl alcohol derivative) is diazotized on release of the side-chain. For IP, this reaction occurs also, but competes with the formation of 6-PNBD-isoproterenol (because the para position of the 3-OH group is unsubstituted), and with redox reactions (characteristic for catechol derivatives) (12). DA does not give rise to 4-PNBD-catechol because the C-1-position, para to the 4-OH-group, is substituted by an alkyl group. VA, VMA, MHPG, normetanephrine, and metanephrine all contain a p-hydroxybenzyl alcohol structure of a p-hydroxybenzoic acid structure, and all are diazotized with release of their alcohol or carbonyl moiety. The only distinction between these metabolites is their different side-chain, but if this side-chain is replaced by a PNBD-group, all the diazonium-derivatives yield the same product, namely 4-PNBD-guaiacol.

In this context, it is also important to know that not all guaiacol derivatives are diazotized to 4-PNBD-guaiacol (7).

In the case of vanillin, the electron-attracting carbonyl group (at the para position of the aromatic hydroxyl group), leads to a decreased electron density at its attachment point, which makes less likely electrophilic azo addition there. No 4-PNBD-guaiacol (violet color) is formed when vanillin is treated with diazotized p-nitroaniline (7).

At acid pH an aromatic OH-group is somewhat less activating than is its conjugated base (O\(^{-}\)). Similarly, a CH(OH)-R group is less electron donating than is its conjugated base. In the case of p-hydroxybenzyl alcohol derivatives, there is decreased preference for azo-substitution at the C-1-position for both of these reasons (13). With compounds possessing a single aromatic hydroxyl group, a slow (and normally masked) reaction, in which azo substitution occurs at the O-position of the aromatic hydroxyl group (by a 6-center mechanism involving one molecule of water), is observed (14). Thus, diazotization of SY, VMA, MHPG, metanephrine and normetanephrine, gives rise to 3-PNBD-symphephrine, 5-PNBD-VA, 5-PNBD-MPG, 5-PNBD-metanephrine, and 5-PNBD-normetanephrine, respectively, along with a decreased formation of 4-PNBD-phenol or 4-PNBD-guaiacol as compared to alkaline pH.

**Clinical Chemical Aspects**

When looking for the presence of meaningful metabolites in urine, such as VMA, one wants no other substances to interfere. A recent report (7) has shown that most of the compounds possessing a guaiacol structure produce the same characteristic "violet" color on treatment with diazotized p-nitroaniline. We have shown here that this is due to the formation of 4-PNBD-guaiacol in the case of VA, VMA, MHPG, normetanephrine, and metanephrine. We show also that this fact is related to the presence of a carbonyl or an alcohol group at the alpha-position of the side-chain of 3-O-methylated catecholamine metabolites. This same configuration seems also necessary to produce a typical blue color when the color test is performed with 2,4-dinitrophenylhydrazine and sodium metaperiodate at an alkaline pH (6).

For all the reasons outlined above, the current spot tests and tube tests (even with ultraviolet spectroscopy) used in screening urine for the presence of secreting neuroblastoma give nearly no reliable information about the presence of increased concentrations of VMA. Thin-layer chromatography of the reaction mixtures after treatment of urine with diazotized p-nitroaniline is also of little use because:

(a) after diazotization at alkaline pH, one can only ascertain the occurrence of 4-PNBD-guaiacol, and not the metabolites from which it is derived.

(b) after diazotization in acid medium, VMA gives rise to another azo derivative besides 4-PNBD-guaiacol, (probably 5-PNBD-VA) that might be used as a "finger print," except that other diazotized compounds in urine may mask or be erroneously taken for this additional spot.

We therefore prefer two-dimensional separation of urinary phenolic acids on microcrystalline cellulose plates. The phenolic acids are identified after spraying
with cold diazotized p-nitroaniline. The details of this method have been published previously (8). On such a chromatogram, catabolites of the catecholamines are discrete, both from each other and from other urinary phenolic acids. Such detailed information allows unambiguous diagnosis of secreting neuroblastoma:

- high excretion of VMA, MHPG, HVA, and iso-HVA in all patients
- high excretion of VA, even when the patients are on a special diet (endogenous VA)
- some patients excrete large amounts of the dopa catabolite VLA, a compound never identified in normal urine, by this method.

Furthermore it is possible to detect excretions of phenolic acids that can lead to false results when other tests are used, e.g., high excretion of VA deriving from vanilla flavoring, high excretion of 3-hydroxy-4-methoxyphenylhydracrylic acid, deriving from hesperidin in the food. A special diet is thus not required.

This chromatographic technique offers the simplicity required for screening methods in the clinical laboratory and it is, in our experience and for the reasons outlined above, more nearly accurate than screening done by using spot tests or tube tests.

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