Differentiation of Urinary N-Acetyl-p-Aminophenol from Endogenous Phenolic Compounds

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An unknown spot, observed on two-dimensional paper chromatograms of extracts of human urine, was identified as N-acetyl-p-aminophenol (APAP), a drug metabolite. This finding suggests that APAP is clearly separable from other endogenous phenolic compounds in urine by a two-dimensional paper chromatographic method. This simple technique is feasible for the specific routine analysis of APAP in urine.

Additional Keyphrases two-dimensional paper chromatography • detection by u absorption and chemical identification • phenacetin

The determination of urinary catecholamine metabolites such as 3-methoxy-4-hydroxy-mandelic acid, homovanillic acid, or 3-methoxy-4-hydroxy-phenylglycol is useful in diagnosing functional neural tumors. The amount of these metabolites in urine is known to be significantly elevated in most cases of these tumors (1, 2).

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Received Oct. 26, 1970; accepted Nov. 18, 1970.


The two-dimensional paper chromatographic system of Armstrong et al. (3) has frequently been used to separate these phenolic metabolites, and the complex mixture of closely related compounds has been found to be well separated on chromatograms by their system (4).

These phenolic compounds are metabolites of endogenous compounds, food constituents, or drugs. Because of the large number of possible compounds, it is sometimes difficult to establish a clear relationship between known and unknown, or uncommon, substances on chromatograms of urinary extracts.

An unknown compound was observed on the two-dimensional chromatograms of extracts of urine from a patient with a neuroblastoma; this paper describes its identification as N-acetyl-p-aminophenol, an analgesic drug.

Materials and Methods

The extract of a urine specimen from a four-year-old white girl with neuroblastoma was submitted to two-dimensional chromatography with use of the solvent system of Armstrong et al. (3). In addition to well-known metabolites, a prominent dark-purple unknown spot was noted on spraying with diazotized p-nitroaniline. Although this spot was observed in extracts of untreated urine, it was much darker on the chroma-
and dinitrophenylhydrazine; nm); solution were been treated with Glusulase; "Glusulase" (8:1:1, by vol) followed by (II) benzene–propionic acid–H₂O (1000:700:41, by vol). The chromatogram was sprayed with diazotized p-nitroaniline. Rf values for the unknown compound were 0.83 and 0.24, respectively.

togram of a pH 7 ethyl acetate extract of enzyme-treated urine (see below) and was located directly above the spot for 3-methoxy-4-hydroxy-phenylglycol (Figure 1).

The unknown spot was isolated as follows: 1200 ml of pooled urine was adjusted to pH 11.5 with 10N NaOH, and saturated barium chloride solution was added until no further precipitate formed. The precipitate was centrifuged, the supernatant fluid was decanted and adjusted to pH 7, then lyophilized. The lyophilized urine was brought to pH 5.5 with 2 ml of 5M acetate buffer, and hydrolyzed by adding 1 ml of "Glusulase" (a mixture of β-glucuronidase and sulfatase, Endo Laboratories, Inc., Garden City, N.Y. 11530) and incubating at 45°C for 18 h. The solution was adjusted to pH 7 with 1N NaOH and extracted three times with three volumes of ethyl acetate.

The pooled ethyl acetate extracts were evaporated under a stream of nitrogen. The residue was dissolved in 2 ml of ethanol and spotted on several sheets (45 X 57 cm) of Whatman no. 3MM filter paper, which had been prewashed with ethanol–water (3:1, by volume). After two-dimensional development in the solvent system of Armstrong et al. (3), the chromatograms were examined under ultraviolet light, and a strongly absorbing area at Rf 0.83 and Rf 0.24 was cut out and eluted with ethanol–water (3:1, by volume). The purity of the eluate was confirmed by rechromatography and gas–liquid chromatography (Figure 2).

**Results and Discussion**

The isolated compound displayed the following properties: (a) the eluate showed absorption maximally at 248 nm (Figure 3); (b) it gave a dark purple solution when reacted with diazotized p-nitroaniline (the maximum absorption of the dye product was at 550 nm); (c) it failed to form vanillin by periodate reaction (5). When a solution of the compound was spotted on filter paper, it gave the following reactions: (d) pink with diazotized sulfanilic acid; (e) no reaction with 2,4-dinitrophenylhydrazine; (f) no reaction with Gibbs' reagent; (g) yellow with p-dimethylaminobenzaldehyde; and (h) no reaction with ninhydrin.

The reaction with p-dimethylaminobenzaldehyde indicated that the compound was a phenylamine, and ruled out the possibility that this unknown compound was a related metabolite of catecholamines. The marked increase after treatment with Glusulase also indicated that the unknown compound was excreted in the urine in conjugated form.

The search was initiated for an exogenous compound with the properties and structure revealed by the above tests. Among several suspected sources, drugs were considered to be the most probable. It was ascertained that the patient had been given aspirin, ascorbic acid, "Tylenol elixir," "Ampicillin," and "Gantrisin" (sulfisoxazole). When these were tested, N-acetyl-p-aminophenol (APAP, acetaminophen, found in Tylenol elixir, McNeil Laboratories, Inc., Ft. Washington, Pa. 19034) was the only compound which had all of the chemical and chromatographic characteristics of unknown.

APAP has been shown to be a major metabolite in urine after ingestion of acethopenetidin (phenacetin).

![Fig. 1. Diagram of two-dimensional paper chromatogram of pH 7 ethyl acetate extract of Glusulase-treated urine. Developed in (I) isopropanol-aq. ammonia–H₂O (8:1:1, by vol) followed by (II) benzene–propionic acid–H₂O (1000:700:41, by vol). The chromatogram was sprayed with diazotized p-nitroaniline. Rf values for the unknown compound were 0.83 and 0.24, respectively.](image)

![Fig. 2. Result of gas chromatography of the unknown. The area of a paper chromatogram containing the unknown compound was eluted with ethanol–water (3:1, by vol) and the eluate was dried and treated with trifluoroacetic anhydride](image)

The derivative was dissolved in ethyl acetate and gas–liquid chromatographed on a 6% QF-1-0055 (60/70 mesh) column at 160°C with a gas flow of 60 ml/min. An electron capture detector (tritium foil) was used at 50 V.

![Fig. 3. Absorption spectra of the unknown compound and of APAP (20 μg) in ethanol–water (3:1, by vol)](image)
It is also available as an analgesic drug, and it is known to be excreted in the urine almost entirely conjugated with glucuronic acid and sulfuric acid.

Clinical laboratories are asked to examine the urine of patients in whom abuse of these analgesic drugs is suspected (7), and various methods of assay have been reported (8,9). These determinations are based on nonspecific chemical reactions. Therefore, separation of APAP from other urinary phenolic compounds by means of two-dimensional paper chromatography of an extract of hydrolyzed urine, as described in this paper, is much more specific. This simple method is feasible for the routine analysis of APAP in the urine in conjunction with spectrophotometry (10).

We thank McNeil Laboratories, Inc. for a sample of N-acetyl-p-aminophenol. This work was supported by grants T-390C from the American Cancer Society, New York, N.Y., and CA08726 from the National Cancer Institute, NIH, USPHS, Bethesda, Md.

References


