Chromatographic Methods for Analysis of the Metabolites of Acetophenetidin (Phenacetin)

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A simple, specific means has been developed for routine analysis of *N*-acetyl-*p*-aminophenol and the other possible metabolites of acetophenetidin in the urine of animals given the drug. The analytical procedures fell into two parts: (*a*) the choice of solvent and pH for extraction and (*b*) the most suitable solvent system for thin-layer chromatography and a selective means for visualization of the compound being assayed. Gas chromatographic separations have also been developed for the analysis of the metabolic transformation products of acetophenetidin.

THE METABOLISM of acetophenetidin[1] has been well studied in man and other animals. It has been shown that most of the dose of acetophenetidin can be accounted for as *N*-acetyl-*p*-aminophenol[2] and its conjugates (1–3). Chemical methods have been developed for the analysis of this phenol in the physiological fluids of animals given acetophenetidin (1, 4).

In the course of studies on the metabolism of acetophenetidin in these laboratories, selective chromatographic methods have been developed for the estimation of the various possible transformation products of acetophenetidin present in urine or other biological material. The advantage of these chromatographic methods is their sensitivity and specificity for a compound present in a multicomponent mixture, but they are not as precise as the chemical methods described. The compound to be estimated is first extracted from buffered solution with a suitable solvent and then subjected to analysis by thin-layer chromatography. More recently, gas chromatographic separations have been developed for more accurate quantitation of the metabolites of acetophenetidin.

MATERIALS AND METHODS

Chemicals—Phenetidin as the free base or the hydrochloride salt was obtained from the Aldrich Chemical Co., Inc., Milwaukee, Wis. *p*-Aminophenol as the free base or the hydrochloride salt was obtained from K and K Laboratories, Inc., Plainview, N. Y. *N*-Acetyl-*p*-aminophenol was supplied by Eastman's Distillation Products Industries, Inc., Rochester, N. Y. 2-Hydroxyacetophenetidin was synthesized in these laboratories by Harfenist (5). Acetophenetidin (Burroughs Wellcome & Company, Tuckahoe, N. Y.) and *N*-hydroxyacetophenetidin were used.[3] All the solvents used were of reagent grade.

Animal Experiments—Acetophenetidin was administered orally in gelatin capsules to 2 adult dogs (8.2 Kg. and 10.2 Kg.) at a level of 100 mg./Kg. for 1 day. A human subject *A* received 420 mg. of acetophenetidin for 1 day. Human subject *B* received 1.8 Gm./day of acetophenetidin for 4 days. Human subject *C* received 1.8 Gm./day of *N*-acetyl-*p*-aminophenol for 4 days. Collections of urine were made for 24-hr. periods during the administration of drugs to dogs and human subject *A*. Urine was collected for the first day (the acute sample) and the fourth day (the chronic sample) of drug administration to human subjects *B* and *C*. Urine collected for the 24-hr. period prior to drug administration was used as control urine.

TABLE I—CHROMATOGRAPHIC EXAMINATION OF URINE

Compd.	Average $Rf \times 100$, System[a] 1	2	3	4	Detection[b]
Acetophenetidin	94	30	80	82	a
Phenetidin	94	70	96	95	a, b, e
N-Acetyl-*p*-aminophenol	88	0	14	34	a, b, c, d
2-Hydroxyacetophenetidin	91	15	55	67	a, b, c, d
N-Hydroxyacetophenetidin	18	0	7	20	a, b
p-Aminophenol	84	0	40	16	a, b, c, d, e

[a] Key to systems: 1, the upper phase of a cyclohexane–propanol–water–acetic acid (20:20:20:1) mixture; 2, the upper phase of benzene–methanol–water–acetic acid (20:10:20:1); 3, the lower phase of ethylene dichloride–methanol–water–acetic acid (20:10:20:1); 4, the lower phase of ethylene dichloride–methanol–water–acetic acid (30:5:10:3). [b] Key to detection: a, ultraviolet absorption; b, alkaline $AgNO_3$ (9); c, Pauly reagent (7); d, diazotized-*o*-dianisidine (8); and e, *p*-dimethylaminocinnamaldehyde reagent (7).

Determinations—The samples to be used for the analysis of acetophenetidin and phenetidin excreted by dogs were prepared by extracting 4 ml. of urine buffered with 1 ml. of glycine buffer at pH 9 (6) and 0.1 ml. of 1 *N* NaOH, with 30 ml. of carbon tetrachloride. A 25-ml. portion of the organic solvent was removed, evaporated, and dissolved in a few drops of methanol. For the measurement of acetophenetidin and phenetidin excreted by human subjects, 8 ml. of urine was treated with 2 ml. of the glycine buffer, 0.25 ml. of 1 *N* NaOH, and extracted with 60 ml. of carbon tetrachloride. A 50-ml. portion of solvent was taken for analysis. The extracts were examined chromatographically with system 2 described in Table I. Phenetidin and acetophenetidin were visualized with ultraviolet light.

A 0.5-ml. portion of urine to be analyzed for *N*-acetyl-*p*-aminophenol was buffered with 0.5 ml. of 0.2 *N* acetate buffer at pH 5 (6) and treated with 0.5 ml. of diluted glusulase[4] (Endo Laboratories, Inc., Garden City, N. Y.) for 4 hr. at 37.5°. The solution was extracted with 15 ml. of ethyl acetate and portions varying from 1 ml. to 5 ml. were taken for analysis. The sample size depended on the concentration of the phenol in urine. The best quanti-

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[1] Phenacetin USP XVII.

[2] Acetaminophen NF XII.

[3] The authors are indebted to Dr. H. C. White, Edgar C. Britton Research Laboratories, Dow Chemical Co., for a supply of *N*-hydroxyacetophenetidin.

[4] To each ml. of glusulase, 1.5 ml. of the acetate buffer was added.

tative results were obtained when the sample applied to a thin-layer plate contained from 10 mcg. to 40 mcg. of phenol (urine highly concentrated with the compound was diluted prior to hydrolysis). After evaporation of the solvent, the residue was dissolved in a few drops of methanol. The *N*-acetyl-*p*-aminophenol was analyzed chromatographically with system 4 described in Table I. The Pauly Reagent (7) was used to visualize the compound.

The analysis for 2-hydroxyacetophenetidin excreted by dogs required hydrolysis of 1 ml. of urine with 1 ml. of the diluted glusulase and 1 ml. of the acetate buffer at pH 5. After hydrolysis, the urine was extracted with 30 ml. of a carbon tetrachloride–methylene chloride (3:1) mixture, and 25 ml. of the solvent mixture was removed. After evaporation of the solvent, the resulting residue was dissolved in a few drops of methanol and examined chromatographically. For the human studies, twice the amounts described above were taken for analysis. The quantification of the hydroxylated acetophenetidin was carried out with system 3 (Table I) and diazotized *o*-dianisidine (8).

To determine the *N*-hydroxyacetophenetidin excreted by human subjects, 4 or 5 ml. of urine was mixed with an equal volume of the acetate buffer and hydrolyzed with 2 ml. of glusulase (nondiluted reagent). The *N*-hydroxy compound was extracted from the hydrolyzed urine with 60 ml. of carbon tetrachloride and a 50-ml. portion of the solvent was taken for analysis. For the hydrolysis of 1 ml. of dog urine, 1 ml. of the acetate buffer and 0.5 ml. of glusulase were added to the urine and 15 ml. of solvent was used to extract the *N*-hydroxyacetophenetidin after hydrolysis. Chromatographic measurements were made on the residue (dissolved in methanol) obtained after evaporation of 10 ml. of solvent, using system 1 (Table I). *N*-Hydroxyacetophenetidin was visualized with ultraviolet light.

Urines obtained prior to drug administration were used as control samples and were treated similarly in all of the above analyses. The reference standards used in the analysis consisted of the appropriate compound added to control urine. Several concentrations, approximating the value of the experimental sample, were made by dissolving the compound in the acetate buffer at pH 5 or glycine buffer at pH 9 and mixing this solution with the urine before hydrolysis or extraction. Standards dissolved in the buffer could be stored at 5° for a reasonable amount of time, but care was taken never to freeze the solutions because of the extremely low solubilities in aqueous solution at the freezing point of most of the above compounds.

Thin-Layer Chromatography—Thin-layer plates were prepared from aluminum oxide GF[5] using techniques previously described (8).

Gas Chromatography—Synthetic mixtures of the above compounds were analyzed on a Barber-Coleman model 10 gas chromatograph equipped with a Sr-90 detector. The inlet pressure was 30 psig for the 6-ft. columns and 25 psig. for the 4-foot columns; the carrier gas was argon. The outlet pressure was atmospheric. The column packings[6] were a mixture of SE-30 (1%)–Carbowax 20M (1%) coated on Anakrom AS, 80–90 mesh, packed in a U-shaped, glass column, 6-ft. in length and 5 mm. i.d., or an SE-30 (0.5%)–Carbowax 20 M (0.5%) mixture on the same support in a column 4-ft. in length and 3.5 mm. i.d.

The instrument was operated isothermally at 180° for the 6-ft. column and at 190° for the 4-ft. column. The detector temperature was always 225° and the temperature of the flash heater was 240°. The samples dissolved in ethyl acetate varied from 0.1 to 0.5 mcg. The retention times are given in Table III.

RESULTS AND DISCUSSION

The analysis for these metabolites in physiological fluids can be accomplished easily using the techniques described. Urines being analyzed for a hydroxylated derivative of acetophenetidin were first subjected to enzymatic hydrolysis to liberate conjugates. All the analyses required the adjustment of the urine to a particular pH value, and a specific organic solvent or solvent mixture was used for extraction. After evaporation of the organic solvent, the residues obtained were chromatographed in one of the solvent systems described in Table I.

The chromatographic systems described appear to be effective in separating several of the reference compounds. However, because of the great differences in the concentrations of the metabolites in the urine and the presence of contaminating substances, a specific system was developed to assay only one particular metabolite. After spraying the developed thin-layer plates, the determinations were made by visual comparison with standards. For precise quantitation, the concentrations of the standards run with each experiment were close to that of the experimental value. These methods have been applied successfully to the analysis of the metabolites of acetophenetidin in the urines of animals treated with the drug or *N*-acetyl-*p*-aminophenol, and the results are given in Table II; the range of the results given in parentheses indicate that this method is not as precise as the methods reported previously, but it is within the usual biochemical variation.

The sensitivities of the methods are given at the bottom of the table. They were not calculated as dose percentages because of the variation in dosages given and urine volumes. The results for the estimation of unchanged acetophenetidin in the urines of animals treated with the drug are in agreement with the values (0.15–3%) reported by Brodie and Axelrod. The method for the estimation of *N*-hydroxyacetophenetidin is the least satisfactory of those described in the experimental section. The amounts usually present were only slightly higher than could be detected with ultraviolet light. The spray reagents tried had high background color.[7] Phenetidin could not be detected in the urines of experimental animals even though a concentration as low as 1 mcg./ml. could be detected in reference samples. Unconjugated *p*-aminophenol is not stable in urine and so could not be estimated by a method which would involve hydrolysis of any conjugates present. The results of the chromatographic estimations confirm *N*-acetyl-*p*-aminophenol as the major metabolite of acetophenetidin and the

[5] Supplied by Brinkmann Instruments Co., Westbury, Long Island, N. Y.

[6] Prepared by Analabs, Inc., Hamden, Conn.

[7] A spray reagent for the visualization of *N*-hydroxylated compounds described by Boyland and Manson (10) appeared since the completion of this work.

TABLE II—THE ESTIMATION OF THE METABOLITES OF ACETOPHENETIDIN BY THIN-LAYER CHROMATOGRAPHY

	Acetophenetidin			*N*-Acetyl-*p*-aminophenol			2-Hydroxyacetophenetidin			*N*-Hydroxyacetophenetidin		
	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose	Urinary mcg./ml.	Concn. Range[a]	Recovery, % of Dose
Human subject *A*	5	4.0–5.6	0.8	350	300–375	73	5	4–7	0.8	0		0
Human subject *B*												
Acute dose	4	3.8–4.1	0.3	500	475–525	62	5	4–7	0.4	10	8–12	0.8
Chronic dose	2	12	0.1	900	850–950	87	20	15–25	1.4	10	6–12	0.7
Human subject *C*[b]												
Acute dose	...	...	...	900	850–950	58	...	...	...	...	...	...
Chronic dose	...	...	...	1200	1100–1230	91.0	...	...	...	...	...	...
Dog 1	16	15–17	0.6	275	250–300	46.0	75	70–80	2.4	10	8–12	0.3
Dog 2	22	20–24	0.9	325	300–350	64	90	85–100	3.4	35	30–40	1.3
Sensitivity of method	0–1 mcg./ml.			Not determined			2–3 mcg./ml.			5 mcg/ml.		

[a] Based on 5 determinations. [b] Treated with *N*-acetyl-*p*-aminophenol.

TABLE III—SEPARATION OF THE METABOLITES BY GAS CHROMATOGRAPHY

	Retention Time, Min.	Column Packing	Length, ft.	Temp.,°C.
Acetophenetidin	13.5	[a]	6	180
	1.2	[b]	4	190
N-Acetyl-*p*-aminophenol	10.0	[b]	4	190
2-Hydroxyacetophenetidin	7.5	[b]	4	190
N-Hydroxyacetophenetidin	13.0	[a]	6	180
	1.1	[b]	4	190
Phenetidin	0.5	[a]	6	180
p-Aminophenol	5.0	[a]	6	180

[a] 1% SE-30, 1% Carbowax 20M. [b] 0.5% SE-30, 5% Carbowax 20 M.

presence of 2-hydroxyacetophenetidin as a minor metabolite (5).

The results are in rough agreement with the earlier studies which employ more time-consuming and less specific methods. There is also present in the urine of animals given acetophenetidin or *N*-acetyl-*p*-aminophenol small amounts of an unknown metabolite with an R_f of 0.35 in solvent system 3[8] which has been reported previously (5).

The analysis of *N*-acetyl-*p*-aminophenol in the urine of animals treated with the tertiary-butyl analog has been reported (11). In this work these separations have been modified and expanded to include acetophenetidin and the other possible metabolites. The separation of acetophenetidin and the related compounds is shown in Table III.

The separation of acetophenetidin from its *N*-hydroxylated derivatives by gas chromatography has not been achieved. Further investigations are continuing in order to find a gas chromatographic means of separating these compounds so that this method can be applied for the analysis of the metabolites of acetophenetidin in the urine of animals given the drug.

[8] It was determined by comparison of the infrared spectrum of this metabolite with the spectrum of 3-hydroxy-acetophenetidin that this compound is not the 3-hydroxylated metabolite.

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Keyphrases

Phenacetin
Metabolites—phenacetin
Urine—phenacetin metabolites, analysis
TLC—analysis
GLC—analysis
IR spectrophotometry—structure