UV Spectrophotometric Method for Determination of Phenacetin in Biological Specimens

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Abstract □ A UV spectrophotometric procedure for determining phenacetin in biological specimens is described. The drug is extracted from biological material by ether and oxidized to a quinone product by cobaltic oxide. The primary metabolite of phenacetin, *N*-acetyl-*p*-aminophenol, is assayed by providing a salting-out step in the extraction process. Better than 90% of phenacetin added to urine and serum specimens *in vitro* at concentrations of 5–50 mcg./ml. was recovered. From homogenized tissue, 83% of added phenacetin was recovered.

Keyphrases □ Phenacetin—UV analysis in biological fluids □ UV spectrophotometry—analysis, phenacetin in biological fluids

Abuse of the analgesic drug phenacetin[1] may lead to certain nephropathies in humans (1, 2). This danger, coupled with the drug's widespread use, necessitates specific and rapid detection of the compound in biological specimens. Phenacetin is a neutral drug that absorbs UV radiation noncharacteristically in the 230–260-nm. portion of the light spectrum. Normal constituents and numerous other pharmacological agents extracted from biological specimens can provide significant interference and make direct UV spectrophotometric analysis of the unchanged drug unrewarding.

Brodie and Axelrod (3) described the metabolic fate of phenacetin in man using an elaborate time-consuming colorimetric procedure. They determined the levels of phenacetin and several metabolites, including *N*-acetyl-*p*-aminophenol and *p*-aminophenol, in biological specimens. Other methods were reported by Welch and Conney (4), Büch *et al.* (5), Cummings *et al.* (6), and Routh *et al.* (7). Each of these techniques is nonspecific, time consuming, or lacking in sensitivity. Prescott (8) reported a sensitive and specific method for the estimation of phenacetin and *N*-acetyl-*p*-aminophenol in plasma and urine by a GLC technique using trimethylsilyl derivatives. The disadvantages of this method are that it has a significant time requisite and absolute dryness is required to achieve an effective silanizing reaction (9, 10).

This report describes a quantitative UV spectrophotometric technique for determining phenacetin in serum, blood, urine, or tissues. Phenacetin is oxidized to a products(s) that has a dissimilar, yet sensitive, response to UV radiation. The phenacetin product is subsequently extracted into sodium hydroxide solution, which results in a highly specific bathochromic shift of the UV spectrum and an increase of sensitivity.

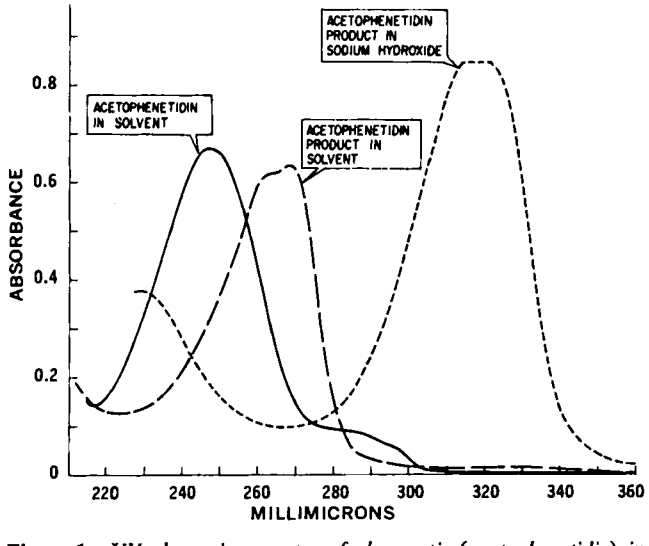

Figure 1—*UV absorption spectra of phenacetin (acetophenetidin) in isopropanol* (——), *the phenacetin product in hexane–*tert*-butanol* (— —), *and the phenacetin product in sodium hydroxide solution* (- - -), *each at a concentration of 8 mcg./ml. corresponding to phenacetin.*

EXPERIMENTAL[2]

Five milliliters of serum or 10 ml. of urine is adjusted to pH 7.0 ± 1.0 and extracted with 50–100 ml. of ether (diethyl). For tissue analysis, 5–10 g. of homogenized tissue is made alkaline (pH 12.0) by addition of several milliliters of 1.0 *N* NaOH and extracted with 100–200 ml. of ether. After extraction the aqueous phase is discarded and the ether layer is washed twice with 20–50 ml. of 1.0 *N* NaOH and once with 20–30 ml. of 1.0 *N* HCl. The organic solvent is filtered through fast-flowing filter paper. The volume recovered is noted and the loss of ether is included in the final calculations.

A major metabolite of phenacetin, *N*-acetyl-*p*-aminophenol, which is itself a commercially available analgesic[3], may be determined by a modification of this procedure. Ten milliliters of urine is adjusted to pH 1.0 ± 0.5 and saturated by the addition of 5 g. of sodium chloride. The original procedure is followed verbatim, except that the two alkali washes of the ether are not performed.

The washed ether is evaporated to dryness under vacuum in a 250-ml. round-bottom flask. Ten milliliters of 2.0 *N* HCl, 20 ml. of a 4:1 mixture of *n*-hexane (spectroquality)–*tert*-butanol, and 200 mg. of cobalt (III) oxide are added to the flask, and the contents are refluxed slowly for 10 min. with constant magnetic stirring. The optimum reflux conditions are achieved by applying 35 v. a.c. to a 270-w. heating mantle containing the flask with attached water-cooled condenser, the mantle being positioned upon a magnetic stirrer. After the flask contents are cooled, the solvent layer is recovered and scanned on the spectrophotometer. A 5-ml. aliquot of the solvent layer is then vigorously extracted with an equal volume of 4.0 *N* NaOH, and the aqueous layer is centrifuged for 5 min. at 3000 r.p.m. and scanned over the 220–360-nm. region; analyses at a single wavelength are achieved by measuring the absorbance at 268 and 320 nm., respectively.

[1] Acetophenetidin.

[2] UV absorption data were obtained with a Beckman DK-2A ratio recording spectrophotometer. A Beckman IR-9 spectrophotometer utilizing KBr disks was used for IR absorption measurements.

[3] Acetaminophen.

Table I—Standard Curve Data of Phenacetin Reaction Product

Phenacetin in Sample, mcg./ml.	Absorbance of Phenacetin Product in Solvent[a]	Absorbance Concentration	Absorbance of Phenacetin Product in Sodium Hydroxide[b]	Absorbance Concentration
10.0	0.798	0.080	1.057	0.106
8.0	0.649	0.081	0.852	0.106
6.0	0.492	0.082	0.623	0.104
4.0	0.321	0.080	0.397	0.099
2.0	0.162	0.081	0.194	0.097

[a] Determined at 268 nm. [b] Determined at 320 nm.

RESULTS

Phenacetin, upon oxidation with cobaltic oxide (Co_2O_3) in dilute hydrochloric acid, was converted to a derivative whose spectrum exhibited maximum absorbance in hexane–*tert*-butanol at 268 nm. and in sodium hydroxide at 320 nm. (Fig. 1). At the levels investigated, a linear relationship exists between the absorbance of the reaction product and the concentrations of drug present (Table I).

Ten milliliters of serum, 10 ml. of urine, and 10 g. of homogenized tissue, to each of which phenacetin was added to provide concentrations of 50, 25, 10, and 5 mcg./ml. (g.), were analyzed; 50 ml. of hexane–*tert*-butanol rather than 20 ml. was used with the 50-mcg./ml. specimens. The recovery of phenacetin by the method of this report is summarized in Table II. Recoveries greater than 90% were obtained from serum and urine, while approximately 83–85% of the drug was recovered from the homogenized liver preparations.

The distribution of phenacetin among the various tissues of the rat, 2 hr. after the animals received 200 mg./kg. body weight of the drug orally, is shown in Table III. Phenacetin, because of low aqueous solubility, was administered to the rats in a suspension of 0.5% methylcellulose in isotonic saline solution. Concentrations of the drug were found in decreasing levels in the following tissues: lungs, brain, kidneys, blood, muscle, and liver. With the exception of the liver, the findings are in agreement with the levels observed by Brodie and Axelrod (3) in dog tissues.

Several drugs, particularly neutral or solvent-soluble agents, were investigated for possible interference (Table IV). The nontherapeutic compound *p*-aminophenol gave an oxidation product with an absorption maximum at 240 nm. in solvent. Extraction of the *p*-aminophenol product into 4.0 *N* NaOH produced a spectrum very similar to that of the phenacetin product. The primary metabolite of phenacetin, *N*-acetyl-*p*-aminophenol, was oxidized by cobaltic oxide to a product with UV spectra (both in hexane–*tert*-butanol and sodium hydroxide solutions) identical to that of the phenacetin derivative. However, *p*-aminophenol and *N*-acetyl-*p*-aminophenol did not interfere with the determination of phenacetin in biological specimens, because both compounds are removed from the final ether extract by the sodium hydroxide wash solution. Compounds other than *p*-aminophenol or *N*-acetyl-*p*-aminophenol provided little or no interference in the determination of phenacetin.

Table II—Recovery of Phenacetin after *In Vitro* Addition to Biological Specimens

Phenacetin Added, mcg./ml.	Recovery[a], Mean[b] ± *SD*		
	Serum	Urine	Liver
50	48.27 ± 1.59	47.31 ± 1.15	43.37 ± 1.08
25	24.26 ± 0.52	22.95 ± 0.78	20.10 ± 0.65
10	9.75 ± 0.24	9.24 ± 0.23	8.30 ± 0.36
5	5.21 ± 0.07	4.71 ± 0.25	—[c]
Average recovery	98.8%	93.2%	83.4%

[a] Determined from readings in 4.0 *N* sodium hydroxide. [b] Average of at least seven determinations. [c] Not determined.

Table III—Distribution of Phenacetin[a] in the Rat

Tissue	Concentration in Tissue: Range of Values, mcg./ml. or g. (Wet Weight)	Concentration in Tissue: Distribution Ratio (Concentration in Tissue/Concentration in Blood), Mean ± *SD*
Blood	20.6–26.4	1.00
Brain	30.8–38.9	1.47 ± 0.12
Kidney	29.4–42.3	1.35 ± 0.13
Lung	31.9–52.9	1.40 ± 0.12
Liver	17.6–20.5	0.70 ± 0.14
Muscle	16.7–24.7	0.92 ± 0.11

[a] Suspension of phenacetin in 0.5% methylcellulose was administered in intraperitoneal doses of 200 mg./kg. body weight, and the animals were sacrificed after 2 hr. Average of determinations for eight rats.

DISCUSSION

Previous methods for the determination of phenacetin in biological specimens are primarily for determining the pharmacological agent in urine and plasma. The method described by Brodie and Axelrod (3) was the only previous report that presented tissue concentrations. Oxidation of phenacetin with cobaltic oxide to a product extractable into aqueous alkali provides a quantitative UV spectrophotometric method for determining the unmetabolized drug in biological specimens. Certain tissues contain large amounts of solvent-extractable material that interfere with the oxidation process. For example, brain tissue is frequently difficult to assay because of its high phospholipid content. The proposed method is applicable to most biological specimens with the exception of fat.

Since *ortho*- and *para*-dihydroxybenzenes, diamines, aminophenols, and related disubstituted benzenes may generally be oxidized to quinones, dependent upon the electronegativity of the substituents and the oxidizing conditions, the method presented in this paper may appear to be quite nonspecific. However, the alkali and acid washes of the ether eliminate all but the most solvent-soluble compounds. Thus, *p*-aminophenol and *N*-acetyl-*p*-aminophenol, very closely related compounds, do not interfere with the analysis of phenacetin. In the absence of solvent washing, considerable specificity is achieved by observing the wavelength of maximum absorbance for different disubstituted benzenes after their oxidation to quinones. Phenacetin and *N*-acetyl-*p*-aminophenol both yield a product absorbing maximally at 268 nm., whereas *p*-aminophenol and *p*-phenetidine apparently yield *p*-benzoquinone which absorbs maximally at 241 nm.

N-Acetyl-*p*-aminophenol, the principal metabolite of phenacetin and itself a commercially available analgesic, is present in high concentrations in the urine. This metabolite is highly water soluble, but it can be determined by the cobalt oxidation technique provided the biological specimen is adjusted to an acidic pH and is saturated with sodium chloride prior to extraction with ether. In addition, the

Table IV—Drugs Found Not to Interfere[a] with the Cobalt Oxide Oxidation Method for Determining Phenacetin[b]

Acetaminophen	Isoniazid
Acetanilide	Meperidine
p-Aminophenol	Meprobamate
Bisacodyl	Methadone
Caffeine	Methenamine mandelate
Carisoprodal	Methyprylon
Chloramphenicol	Morphine
Chlormezanone	Phenacemide
Chloroquine	Phenaglycodol
Dicumarol	Phenylbutazone
Diethylpropion	Phenylephrine
Ethchlorvynol	Propoxyphene
Ethinamate	Salicylamide
Glutethimide	Salicylic acid
Hydroxyphenamate	Trimethadione

[a] Absorbance of the oxidation products in sodium hydroxide was less than 0.01 for 10 mcg./ml. of each drug. [b] Absorbance measured at 320 nm.

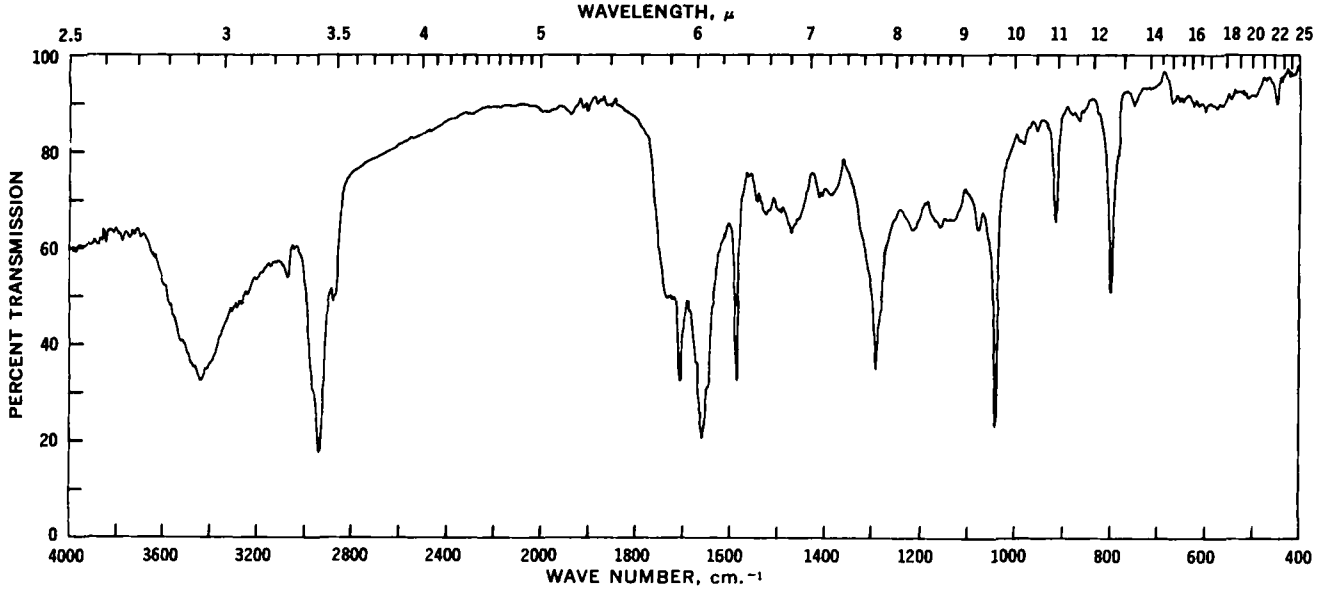

Figure 2—*IR absorption spectrum of the cobaltic oxide oxidation product from phenacetin.*

alkali wash steps must be eliminated to prevent the removal of metabolites from the ether solvent.

IR data indicate that the phenacetin oxidation product(s) consists of a carbonyl group, as evidenced by the strong absorbance (Fig. 2) in the 6-μ region. Aliphatic C—H stretching was conspicuous at 3.4 μ. The phenacetin product(s) is suggested to be a mixture of quinone- or quinonimine-type compounds with aliphatic substituents. The exact chemical structure has not been elucidated for the product(s) but this information is not essential for routine application and effectiveness of the procedure.

SUMMARY

Biological specimens can be analyzed spectrophotometrically for phenacetin after oxidation of the drug by cobaltic oxide. The procedure has an excellent amount of specificity, with no other group of pharmacological agents affording interference. The particular extraction technique required to recover the neutral drug phenacetin from biological environments makes specimens with high lipid concentrations difficult to analyze.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 11, 1972, from the **Department of Pathology, University of Texas Medical School at San Antonio, San Antonio, TX 78229*, the *†Forensic Toxicology Branch, USAF School of Aerospace Medicine, Aerospace Medical Division, Brooks Air Force Base, TX 78235*, and the *‡Department of Pharmacology, University of Texas Medical School at San Antonio, San Antonio, TX 78229*

Accepted for publication October 20, 1972.

The research reported in this paper was conducted by personnel of the Forensic Toxicology Branch, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, United States Air Force, Brooks AFB, Tex. Further reproduction is authorized to satisfy the needs of the U. S. Government.

The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care," published by the National Academy of Sciences–National Research Council.

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