ACKNOWLEDGMENTS AND ADDRESSES

Received June 17, 1974, from the *Food and Drug Administration, Department of Health, Education, and Welfare, 850 Third Avenue, Brooklyn, NY 11232*

Accepted for publication October 9, 1974.

Adapted in part from a thesis submitted by S. Fishman to the Graduate Division, Brooklyn College of the City University of New York, in partial fulfillment of the Master of Arts degree requirements.

The author thanks Dr. Thomas Medwick, Science Advisor, Food and Drug Administration, New York District, and Professor of Pharmaceutical Chemistry, Rutgers University, New Brunswick, N.J.; Dr. T. S. Ma, Dr. R. Ginell, and Dr. J. Glickstein, Professors of Chemistry, Brooklyn College, Brooklyn, N.Y.; and William M. Plank, Research Coordinator, Food and Drug Administration, New York District, for their advice and assistance in the preparation of this paper. The author also thanks Dr. Donald Williams, Ayerst Laboratories, Rouses Point, N.Y., for his donations of estrogen standards.

The research leading to publication of this paper was pursued under the Food and Drug Adminstration's Science Advisor's Research Associate Program.

Determination of Acetaminophen in Pharmaceutical Preparations and Body Fluids by High-Performance Liquid Chromatography with Electrochemical Detection

RALPH M. RIGGIN, ALLEN L. SCHMIDT, and PETER T. KISSINGER ˣ

Abstract □ A sensitive and very rapid assay for acetaminophen was developed based on the combination of high-performance chromatographic columns with a thin-layer electrochemical detector. Application to liquid and solid dosage forms and body fluids has been demonstrated. Great advantage derives from the detector selectivity, which permits discrimination against many potentially interfering substances without need for extensive separations or formation of derivatives. As little as 0.005% of the hydrolysis product, *p*-aminophenol, can be detected in the presence of the intact drug following cation-exchange chromatography. Acetaminophen can be quantitatively determined in serum on the 50-ng/ml level by liquid chromatography using a pellicular polyamide packing.

Keyphrases □ Acetaminophen—analysis in dosage forms, serum, and urine, high-performance liquid chromatography with electrochemical detection □ Liquid chromatography, high performance—determination of acetaminophen in pharmaceutical preparations and body fluids, electrochemical detection □ *p*-Aminophenol—determination of trace amounts in acetaminophen preparations, high-performance liquid chromatography with electrochemical detection

Fundamental advances recently have been made in liquid chromatography and analytical electrochemistry. Both fields depend on heterogeneous processes, which are now sufficiently well understood to permit optimization of the controlling parameters. This knowledge has resulted in a new enthusiasm for methods that only a few years ago were thought to be outmoded. Liquid chromatography, while easy enough to use, continues to suffer from the lack of a completely satisfactory detector, particularly one with sufficient sensitivity to compete with GC. On the other hand, modern electrochemical techniques have attained considerable sensitivity but suffer from rather poor resolution, chronic irreproducibility, and poor human engineering.

It is not surprising that great advantages accrue from combining the two technologies. Strong parallels exist with the more established association of liquid chromatography and electronic spectroscopy (both absorption and emission). In both cases, two phenomena of modest specificity are combined into a single high-resolution device. The optical instrumentation presently used in liquid chromatography is often insufficiently sensitive or selective for quantitating body fluid levels for many clinically important compounds. Although less generally applicable than optical devices, electrochemical detectors can sometimes form the basis for successful assay procedures.

Recently, a thin-layer electrochemical transducer was developed that has significant advantages over conventional electrochemical cells when coupled to high-performance liquid chromatography (1, 2). The principles of operation of this detector and other developments in hydrodynamic electrochemistry have been described (2, 3). In brief, the column effluent is passed between two plates separated by a thin spacer (typically 50 μm). Electrodes imbedded in the walls of the channel efficiently convert sample molecules into the product due to the short diffusional pathway across the moving solution film. It is possible in this manner to detect as little as 1 pg of an electroactive material while maintaining a detector dead volume of less than 1 μl.

In view of the large number of electrochemically reactive pharmaceuticals, liquid chromatography with electrochemical detection could have wide utility in quality control and drug metabolism studies. The present paper describes several procedures developed for acetaminophen (*N*-acetyl-*p*-aminophenol) in dosage forms and body fluids.

Acetaminophen is most commonly assayed in dosage forms by spectrophotometry. The drug may be monitored directly (4, 5) or following conversion to 2-nitro-4-acetamidophenol (6). Many published procedures are based on acid hydrolysis to *p*-aminophe-

nol followed by oxidative coupling with a phenol to an indophenol dye (7). More recent approaches include stationary electrode voltammetry at a glassy carbon electrode (8) and nonaqueous potentiometric titrimetry with tetrabutylammonium hydroxide in dimethylformamide (9). None of these procedures is suitable for the determination of trace quantities of the *p*-aminophenol hydrolysis product in the presence of the intact drug.

Many papers have described the determination of total acetaminophen in body fluids by initial hydrolysis of conjugates as well as the free drug to *p*-aminophenol. This common derivative is extracted and subsequently coupled to form an azo or indophenol dye suitable for spectrophotometry (10, 11). More specific and generally more sensitive procedures are based on GLC of the free drug (12, 13) or its trimethylsilyl derivative (14). Studies of phenacetin and acetaminophen metabolism by high-resolution liquid chromatography with UV detection, whereby individual metabolites were identified and quantitated, were reported (15, 16).

EXPERIMENTAL

Reagents and Materials—Distilled water, deionized and finally distilled from alkaline potassium permanganate, was used for preparing all solutions. Acetaminophen, *p*-aminophenol, phenacetin, and salicylamide were obtained from commercial sources. *p*-Aminophenol was purified by vacuum sublimation at <1 mm Hg and 120°. Tablet and liquid dosage forms were purchased locally. All other chemicals were reagent grade and were not further purified.

Glass chromatographic columns[1], 30 cm × 2 mm i.d., were dry packed with pellicular strong cation-exchange[2] or polyamide[3] high-performance packings. Dilute sulfuric acid (0.04 *M* at 0.25 ml/min) was used as the eluent for the cation-exchange column, and phosphate buffer (0.04 *M*, pH 7.4 at 0.35 ml/min) was used for the polyamide column.

Standard solutions of acetaminophen were prepared by diluting a stock solution (1.0 mg/ml) in 0.04 *M* H_2SO_4. Instrumentation was calibrated using six solutions between 10 and 80 ng/μl. Standard solutions containing 200 ng/μl of acetaminophen and between 10 and 400 pg/μl of *p*-aminophenol (*i.e.*, 0.005–0.2%) were prepared in distilled water and used to calibrate the cation-exchange system for trace *p*-aminophenol.

Apparatus—Chromatographs were assembled from commercially available modular components, and the electrochemical detector cell was constructed as described previously (2). The instrumentation used to control the applied potential and to measure the resulting current is depicted in Fig. 1. A conventional three-electrode configuration is used with auxiliary, reference, and working electrodes of platinum, silver–silver chloride, and carbon paste, respectively. The currents encountered in detection of trace organics in the eluate from high-performance liquid chromatography are frequently below 1 namp. Therefore, it is essential to use operational amplifiers with low bias currents for OA-1 and OA-2[4] and to guard their inputs against stray leakage currents along the circuit board. The requirements for OA-3 and OA-4 are more modest; standard 741-type units will suffice.

SW1 and R14 permit selection of the appropriate applied potential. SW3 and SW4 provide a selection of the gain and filtering in the first stage of amplification. SW5, R15, OA-4, and R5 provide a balance for the standing background current encountered in the absence of an eluted zone. When SW3 is in the most sensitive position, SW6 provides a range of current sensitivities of 0.5, 1, 5, 10, 50, and 100 namp/v. The values for the passive components used are as follows: R1, 33K; R2, 100K; R3, 1M; R4, 10M; R5–R8, 1K; R9, 2K; R10, 10K; R11, 20K; R12, 100K; R13, 200K; R14, 5K; C3, 1 μF; C4, 0.47 μF; and C5, 0.01 μF.

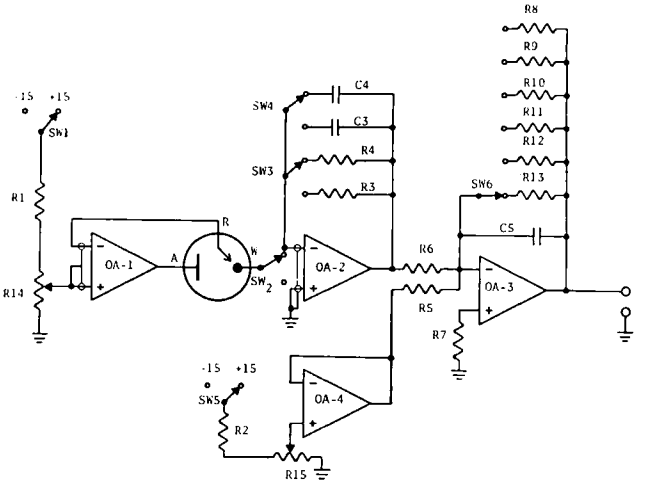

Figure 1—*Electronic instrumentation for electrochemical detection. Key: A, auxiliary electrode; R, reference electrode; and W, working electrode.*

All quantitative measurements were accomplished by analog integration of the output from OA-4 using an operational amplifier integrator with a time constant of 10 sec. The results were displayed on a digital voltmeter. The integrated response can be converted directly into the microcoulombs of charge passed for a given chromatographic peak. By using standard solutions, the amount of charge required for complete conversion can be calculated from Faraday's law. Measurements at 0.25 ml/min mobile phase velocity indicate that about 10% conversion is typical for low molecular weight phenolic compounds such as acetaminophen. Higher conversion is possible at lower flow rates or for cells with thinner channels and/or larger electrodes; however, such higher conversion does not contribute significantly to either sensitivity or precision.

The linear range of calibration plots of charge *versus* injected amount is dependent on cell geometry due to the uncompensated resistance along the thin-layer channel. For a 50-μm cell and a flow rate of 0.025 ml/min of a 0.1 *M* mobile phase electrolyte, a significant negative deviation typically occurs above several hundred nanograms for molecules with short retention times. If very large samples (*e.g.*, several micrograms) are to be injected, linearity of response can be ensured by adjusting the cell thickness up to 200 μm and/or increasing the applied potential. In general, it is more satisfactory to dilute the sample to within the established linear range.

Current–voltage curves (*i.e.*, voltammograms) for oxidation of acetaminophen and other phenolic compounds were determined using a carbon paste electrode of 3 mm diameter packed in a Teflon well with an outside diameter of 7 mm. The carbon paste was of the same composition described earlier (1). A simplified circuit using three integrated operational amplifiers was used in association with small volume (~10 ml) Pyrex cells, with the electrodes positioned in a Teflon cap[5].

Procedure—Five commercial products, as well as serum and urine samples, were analyzed for acetaminophen. Samples (2 μl) prepared according to the following procedures were injected manually onto the chromatographic columns through silicone septa using 5-μl syringes[6]. The peak areas were converted into nanograms injected by comparison with the least-squares slope determined from the six calibration standards described. The applied potential was +1.0 v *versus* a silver–silver chloride reference electrode (3 *M* NaCl) for the acetaminophen determination.

Preparation of Solid Dosage Forms—Five tablets were accurately weighed and then pulverized to a fine powder. A known per-

[1] Model MB-2-300, Chromatronix, Inc.
[2] Zipax SCX Part No. 820950002, E. I. DuPont de Nemours & Co.
[3] Pellidon, H. Reeve Angel & Co.
[4] National semiconductor LH0042CH.

[5] Photographs and construction details for locally built apparatus are available on request.
[6] Hamilton 85-N.

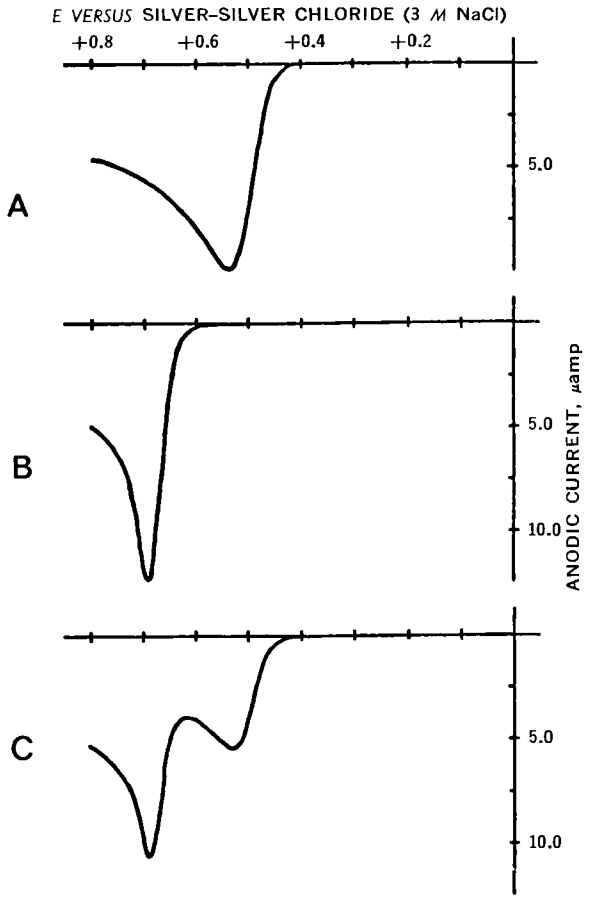

Figure 2—*Stationary electrode voltammetry (0.14 v/sec) at the carbon paste electrode* (d_w = *3 mm*) *in 0.1* M *$HClO_4$. Key: A, 1 m*M p-*aminophenol;* B, *1 m*M *acetaminophen* (N-*acetyl*-p-*aminophenol*); *and C, 0.5 m*M p-*aminophenol and 0.5 m*M *acetaminophen.*

centage of the total mass (1–4%) was then weighed and diluted to 100 ml with 0.04 *M* H_2SO_4. The solution was shaken for 5 min to ensure complete dissolution of acetaminophen. A 10-ml aliquot was transferred to a second 100-ml volumetric flask and diluted with 0.04 *M* H_2SO_4. The resulting solution, containing from 10–40 ng/μl, was used for the analysis.

Preparation of Liquid Dosage Forms—Two milliliters of the liquid product was diluted to 100 ml with 0.04 *M* H_2SO_4. A 10-ml aliquot of this initial solution was diluted again to 100 ml with 0.04 *M* H_2SO_4 to yield a final concentration in the 20–30-ng/μl range.

Determination of Trace p-*Aminophenol in Acetaminophen*—The initial solutions prepared from the dosage forms contained 100–400 ng/μl acetaminophen. These more concentrated solutions were analyzed for *p*-aminophenol. To monitor trace amounts of *p*-aminophenol successfully on the level of 0.005–0.2% of the acetaminophen present, it is necessary to decrease the response of the detector to the latter compound by lowering the applied potential. A potential of +0.65 v was satisfactory for this purpose. Therefore, it was possible to detect as little as 0.005% of *p*-aminophenol in the original dosage forms.

Determination of Acetaminophen in Serum—Three-milliliter aliquots from a normal serum pool were placed in 50-ml polyethylene centrifuge tubes containing 2 g of sodium chloride and 3 ml of saturated sodium chloride. The samples were spiked with standard acetaminophen over a 0.1–10-μg/ml range. Concentrated perchloric acid (0.5 ml) was added, and the tubes were mixed on a vortex mixer for 30 sec. Following centrifugation at 14,000 rpm for 15 min, 5 ml of the supernate was transferred to a 15-ml glass centrifuge tube. After adjustment to pH 7.0, the solution was extracted with two 5-ml portions of ethyl acetate.

The ethyl acetate layers were combined in a second 15-ml centrifuge tube and back-extracted with a single 2-ml portion of pH 7.0 McIlvaine buffer to minimize the residual uric acid. The ethyl acetate layer was then evaporated to dryness at 40° under a stream of nitrogen. The residue was brought up in 200 μl of 0.04 *M* H_2SO_4 with thorough mixing, and then 2 μl was injected onto the polyamide column and detected at a flow rate of 0.35 ml/min and a potential of +0.80 v *versus* silver–silver chloride. It is easy to make the measurement on 1 ml of serum with little sacrifice in precision by proportional adjustment of the volumes used in the extraction procedure.

The polyamide column was used to minimize interference from uric acid carried through the extraction procedure, since it is easily oxidized at a carbon electrode (17, 18). Because uric acid is not adequately resolved from acetaminophen on the cation-exchange resin, it remains a possible interference in trace determinations of the latter compound in serum and urine. Fortunately, acetaminophen is more strongly retained than uric acid on the polyamide column. The chromatographic resolution adequately compensates for the poor electrochemical selectivity for the two compounds.

Determination of Acetaminophen in Urine—The procedure was identical with that described for serum, except that 5 ml of sample was used without the addition of the sodium chloride solution. Back-extraction of the ethyl acetate with pH 7 buffer was repeated twice due to the 10-fold greater amount of uric acid present in urine. Recovery of standard additions to urine was typically 83%.

RESULTS AND DISCUSSION

The performance of an electrochemical detector can be optimized on the basis of preliminary voltammetric studies. The compounds of interest and possible interferences are examined to establish the potentials sufficient to react them at a diffusion-controlled rate. Figure 2 illustrates behavior typical of phenolic amines in acidic media. The electrochemical process involves a two-electron conversion of the compounds to intermediate species which eventually react to form benzoquinone. This chemistry has not yet been worked out for acetaminophen; however, a detailed study is in progress. Acetaminophen and *p*-aminophenol have oxidation potentials different by about 150 mv in acid solution. When the two compounds are present in a mixture at similar concentrations, they may be clearly distinguished as in Fig. 2C.

Quantitative estimation of both compounds based on the peak currents is difficult because the *p*-aminophenol wave overlaps with that due to acetaminophen in a manner not readily accounted for

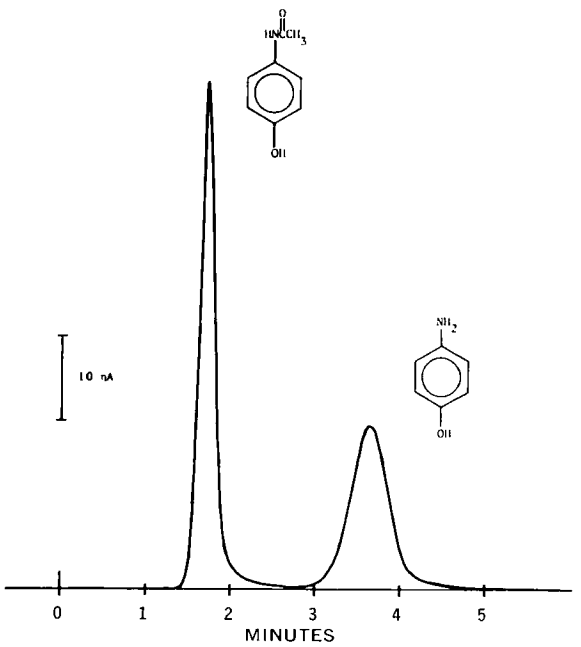

Figure 3—*Liquid chromatography of 7.5 ng acetaminophen* (N-*acetyl*-p-*aminophenol*) *and 7.5 ng* p-*aminophenol* (E = + *1.20 v* versus *silver–silver chloride 30* × *0.2-cm column, 0.25 ml/min 0.04* M *H_2SO_4*).

Table I—Analysis of Dosage Forms Containing Acetaminophen

Dosage Form	Labeled Composition, mg/Dose	Measured Composition, mg/Dose	Percent of Labeled Value Found
A (tablet)	388	373 ± 0.50%[a]	96.1
B (tablet)	194	187 ± 1.2%	96.3
C (tablet)	385	375 ± 0.41%	97.4
D (liquid)	120	121 ± 1.1%	101
E[b] (liquid)	600	589 ± 0.14%	98.2
F (liquid)	600	577 ± 0.60%	96.1
G (liquid)	600	582 ± 0.12%	97.0

[a] Relative standard deviation based on at least five determinations. [b] Liquids E, F, and G were different samples of the same dosage form purchased over a 1-year period.

mathematically. Because the heterogeneous reaction of acetaminophen is exponentially dependent on potential, at potentials near the foot of the wave in Fig. 2B, it is nearly impossible to determine trace quantities of *p*-aminophenol in the presence of a much larger amount of acetaminophen since both reactions occur to a significant extent at the peak potential of the former molecule. The problem of resolving successive electrochemical reactions is an inherent disadvantage of voltammetric methods, particularly since all compounds react at the potentials needed to quantitate the least reactive component. Since the hydrolysis of acetaminophen is a very slow reaction at ordinary temperatures (19), *p*-aminophenol is commonly present at well below 1% in dosage forms. A voltammetric approach to analysis of this undesirable decomposition product is clearly insufficient.

Physical separation of the two components is readily accomplished *via* high-performance cation-exchange chromatography (Fig. 3). From the voltammetric information, a potential of about +0.70 v or greater is sufficient to detect both compounds using electrochemical detection. Therefore, it is easily possible to make quantitative measurements on both compounds at the 100-pg level, well below the detection limit for presently available UV instruments. Nevertheless, determination of trace *p*-aminophenol is still impossible when acetaminophen is present in a 1000-fold excess. Fortunately, this problem can easily be overcome by operating the detector at lower potentials so that the response is selective to *p*-aminophenol. The same degree of selectivity is not possible with a UV detector since the absorption spectra of the two compounds are insufficiently resolved.

The results from an analysis of several popular dosage forms containing acetaminophen are given in Table I. All were found to be within the regulated specifications (20). Other components in the preparations were eluted at different retention times and/or were not electroactive. Several compounds (*e.g.*, salicylamide and phenacetin) also present as active ingredients were much more difficult to oxidize than acetaminophen and could be easily "tuned out" by adjusting the potential applied to the detecting electrode. In each sample, the *p*-aminophenol level was below 0.01% of the label value for acetaminophen, well within the regulated maximum of 0.25% for the bulk drug. It is believed that the present method for monitoring trace *p*-aminophenol is superior to the standard semiquantitative TLC assay (20).

The determination of free acetaminophen in biological fluids was easily accomplished by simple extraction following protein precipitation. In serum the response was linear over the range tested (0.1–10 μg/ml), and quantitative data (typically to about ±10% *RSD*) could be obtained on a level an order of magnitude below the capability of GC procedures (13, 14). In addition, this simple method is far less time consuming and more precise than those involving derivative formation (14).

The assay of acetaminophen in urine is more difficult than serum, primarily due to the larger concentration of uric acid. The limit for quantitative measurement is at present about 1 μg/ml. The clearance of free drug was found to be 2.1% over 24 hr for a 22-year-old male following a single 1-g dose of a commercial elixir. Conjugated metabolites (glucuronides and sulfates) can be estimated following acid or enzymatic hydrolysis in the usual manner. It is hoped that conjugated phenols derived from aromatic drugs will be able to be detected without prior hydrolysis. Recent work in this laboratory indicates that many of these are electrochemically active.

In conclusion, high-performance liquid chromatography with thin-layer electrochemical detection provides unusual sensitivity and selectivity at minimum expense. Besides acetaminophen and related molecules, many pharmaceutical products are suitable for analysis in this way (21, 22). Isoproterenol, levodopa, α-methyllevodopa, norepinephrine, epinephrine, dopamine, phenylephrine, and chloramphenicol are among the compounds that have been successfully analyzed. The applicability of the technique can be greatly expanded by nitration (23) or hydroxylation (24) of compounds containing a nonelectroactive phenyl moiety.

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ACKNOWLEDGMENTS AND ADDRESSES

Received July 22, 1974, from the *Department of Chemistry, Michigan State University, East Lansing, MI 48824*

Accepted for publication October 11, 1974.

Supported in part by Grant GP-42452X from the National Science Foundation.

[x] To whom inquiries should be directed.