High-Voltage Paper Electrophoresis for Characterization of Drug Metabolites

WALTER D. CONWAY[x], VIJAY K. BATRA, and ALAN ABRAMOWITZ

Abstract ☐ Methodology for obtaining reproducible measurements of the relative mobility of organic compounds over pH 2–12 using high-voltage paper electrophoresis is described. Use of the method for the elucidation of some structural features of simple molecules is illustrated by applying it to the characterization of metabolites of drugs excreted in the urine and bile of rats. The compounds studied included p-nitrophenol, 1-naphthol, salicylic acid, 4-nitrocatechol, probenecid, phenol red, and their metabolites.

Keyphrases ☐ Mobility–pH profiles, drug metabolites—determined using high-voltage paper electrophoresis ☐ Metabolites—characterized using high-voltage paper electrophoresis ☐ Structure characterization—mobility–pH profiles of drug metabolites determined using high-voltage paper electrophoresis ☐ Paper electrophoresis, high voltage—determination of mobility–pH profiles for drug metabolites

Paper electrophoresis has been widely used over the past 20 years for separating and characterizing molecules. An historical account and an extensive review of the work done prior to 1955 was presented by McDonald (1). The technique has been extensively applied in the fields of carbohydrate chemistry, amino acid and protein analysis, and inorganic chemistry.

Recent applications related to drugs include separation and identification of alkaloids (2); establishment of the radiopurity of ¹⁴C-labeled methylglyoxal-bisguanyl-hydrazone; and separation of the metabolites of chlorpromazine (5, 6), carbiphene hydrochloride[1] (a basic amide analgesic) (7), catecholamines (8–10), isoproterenol (11), derivatives of fluorene and biphenyl (12), p-aminohippuric acid and its acetyl derivatives (13), cyclophosphamide (14), thalidomide (15), neostigmine (16, 17), and pronethalol (18).
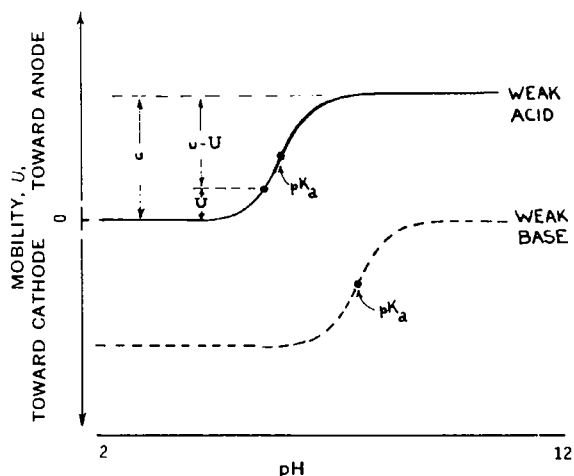

Figure 1—*Mobility–pH profiles of a weak acid and a weak base.*

Bond and Howe (18) classified the metabolites of pronethalol as acidic, basic, and amphoteric on the basis of electrophoretic mobility at a single pH; in the same way, Keberle et al. (15) classified thalidomide metabolites as monocarboxylic and dicarboxylic acids. Most investigators made separations in only one or two buffers and applied electrophoresis in much the same manner as other differential migration techniques such as TLC and paper chromatography. However, significant aspects of the structure of a substance can be ascertained simply from measurements of its electrophoretic mobility at several concentrations of hydrogen ion. This technique is particularly useful for determining structures of substances arising from drug metabolism.

THEORETICAL

The relationship of various factors affecting the mobility, U, of a spherical particle in an electrolyte under the influence of an electrical field was expressed (19) as Eq. 1:

$$U = \frac{Q}{6\pi\eta r}\left(\frac{1}{1 + \kappa r}\right) \qquad \text{(Eq. 1)}$$

where Q is the electrical charge on the particle, r is its radius, η is the viscosity of the electrolyte, and κ is a parameter, the reciprocal of which is related to the thickness of the ionic double layer surrounding the particle. The equation implies that doubly charged ions migrate twice as fast as singly charged ions of equal size. Where $1/\kappa$ is small compared with r, a reasonable approximation in the case of organic drugs, there is an inverse dependence of mobility on the square of the radius of the particle.

The general shape of a plot of mobility *versus* pH for a weak monoprotic acid is characterized by a form of the Henderson–Hasselbalch equation:

$$pH = pKa + \log\left(\frac{U}{u - U}\right) \qquad \text{(Eq. 2)}$$

and can be advantageously illustrated as shown in Fig. 1. It is convenient to refer to this plot as the mobility–pH profile of a substance.

The shape of the mobility–pH profile is, in fact, identical with that of a plot of the ionized fraction of a monoprotic acid as a function of pH since it can be shown that:

$$\frac{U}{u - U} = \frac{\alpha}{1 - \alpha} \qquad \text{(Eq. 3)}$$

where U is the mobility of a zone at any particular pH, u is the maximum mobility of the zone or the mobility of the anion in the case of a monoprotic acid, and α is the degree of dissociation of the weak acid (20). The term α may be obtained from the graph at any particular pH as the ratio of the distances U and u since:

$$\alpha = \frac{U}{u} \qquad \text{(Eq. 4)}$$

The degree of dissociation, α, may also be interpreted as that fraction of time spent in the ionized state by any individual molecule of a weak acid. The fraction varies from 0 at low pH to 1 at higher pH, where the region of constant mobility is reached. This interpretation of α is advantageous in rationalizing the shape of the mobility–pH profile.

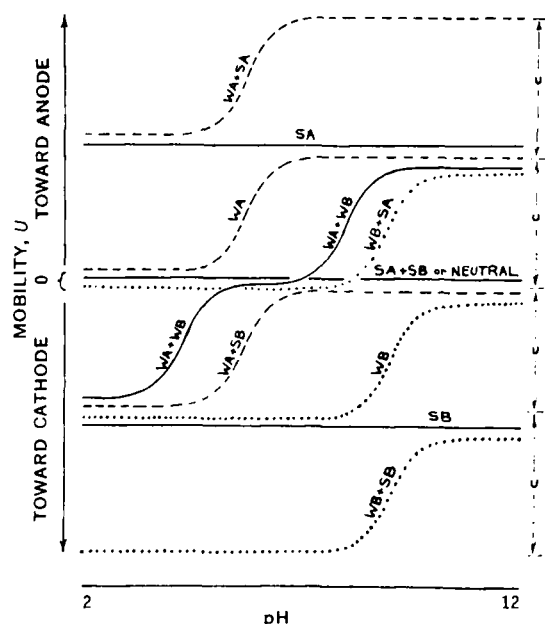As indicated by Eqs. 2 and 3, the profile of a weak monobasic

Figure 2—*Mobility-pH profiles of weak acids (WA), weak bases (WB), strong acids (SA), strong bases (SB), and amphoteric molecules containing combinations of these functional groups.*

acid will have: (*a*) zero mobility at low pH, where the acid is not ionized and is, therefore, electrically neutral; (*b*) a point of inflection where the pH of the electrolyte equals the pKa of the weak acid; and (*c*) a constant mobility toward the anode at high pH, characteristic of the anion resulting from ionization of the acid. The distance *u* will be related to the effective radius or volume of the ion. Under conditions of zero ionic strength, and assuming hydrodynamic behavior of ions equivalent to that of spheres, the distance *u* would be expected to be inversely related to the two-thirds power of the molecular weight.

The profile of a weak base can be described in completely analogous fashion, using Eq. 2, by considering the protonated form of the base to behave as a weak monoprotic acid, albeit bearing a unit positive charge at low pH. The weak base will, therefore, exhibit a constant maximum mobility toward the cathode in the region of low pH and zero mobility at high pH (Fig. 1).

The *shape* of the mobility-pH profile of either a weak acid or a weak base is thus defined by Eq. 2. The mobility increment, *u*, will depend upon the molecular weight of the substance, and the *position* of the sigmoid portion of the profile will be determined by the pKa of the substance.

Strong acids, such as sulfonic acids and sulfuric acid monoesters, will migrate toward the anode, and completely dissociated cations, such as quaternary ammonium compounds, will migrate toward the cathode at constant velocity independent of pH. The profile of an amphoteric substance should reflect the combined behavior of the acid and base functions.

The general features of the mobility-pH profiles predicted by Eq. 2 for molecules containing various combinations of strong and weak acid and base functional groups are illustrated in Fig. 2. The profiles are drawn for compounds of equal molecular weights and arbitrary pKa values. For clarity, the curves are shifted slightly on the vertical axis from their normal positions, which would overlap in the regions of constant mobility.

A measure of the pH interval over which ionization of a substance occurs may be obtained by drawing a line through the relatively linear portion of the mobility-pH profile and extrapolating it to its intersections with those portions of the profile that define regions of constant minimum and maximum mobility. It is useful to refer to this pH increment as the ionization interval. The relationship of this ionization interval to the shape of the mobility-pH profile may be seen by differentiating Eq. 2 with respect to pH and evaluating the resulting equation in terms of *u* at the point of inflection or pKa, where $U = u - U = u/2$, which gives:

$$\left[\frac{dU}{d(\text{pH})}\right]_{\text{pKa}} = \frac{U(u - U)}{U \log_{10} e} = \frac{u}{1.74} = \text{slope at pKa} \quad \text{(Eq. 5)}$$
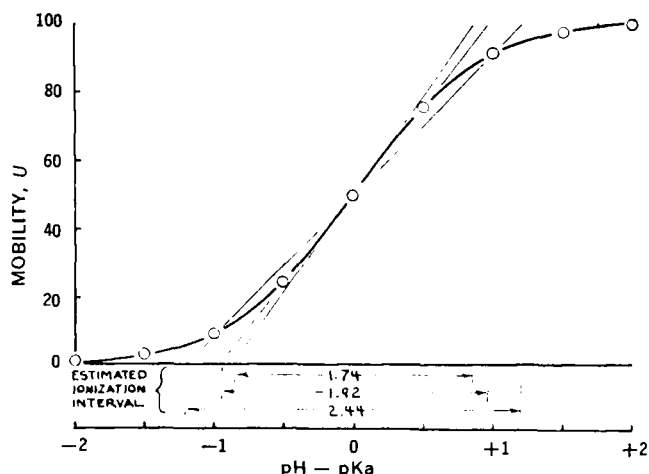

Figure 3—*Estimates of the ionization interval obtained from lines drawn tangent to the inflection point and through points on the mobility-pH profile at pH − pKa = ±0.5 and ±1.0.*

This implies that, irrespective of molecular weight, a line drawn tangent to a continuous plot of the mobility-pH profile at the pKa will intercept the values of minimum and maximum mobility with an intervening distance of 1.74 pH units for a monoprotic acid or base (Fig. 3). However, a line drawn through discontinuous experimental data points obtained at increments of 0.5-1.0 pH unit will have a slightly lower slope. For instance, the line drawn through points spaced 0.5 pH unit above and below the pKa will form intercepts with the minimum and maximum mobilities with an intervening distance of 1.92 pH units, and the intercepts derived from a line through points spaced 1.0 pH unit above and below the pKa will be 2.44 units apart (Fig. 3).

A diprotic acid will possess two electrical charges when fully ionized. The acid will be 50% ionized at a hydronium-ion concentration, [H⁺], given by Eq. 6:

$$[H^+] = \sqrt{K_1 K_2} \quad \text{(Eq. 6)}$$

where K_1 and K_2 are the apparent dissociation constants of the two ionizable groups. In logarithmic form, Eq. 6 becomes:

$$\text{pH} = \frac{pK_1 + pK_2}{2} \quad \text{(Eq. 7)}$$

The ionization and, consequently, the electrophoretic mobility of a diprotic acid may be conveniently expressed as:

$$\text{percent ionized} = 100 \left(\frac{U}{u}\right) = 50 \left(\frac{\Delta\sqrt{X} + 2}{\Delta^2 + \Delta\sqrt{X} + 1}\right) \quad \text{(Eq. 8)}$$

where:

$$\Delta = \frac{[H^+]}{\sqrt{K_1 K_2}} \quad \text{(Eq. 9)}$$

$$X = \frac{K_1}{K_2} \quad \text{(Eq. 10)}$$

The mobility of a zone of a diprotic acid at a particular pH will be proportional to the charge on the acid at that pH. As described by Eq. 8, the profile will be symmetrical about the point where the acid is 50% ionized. At this point, $\Delta = 1$ or $\text{pH} = \frac{1}{2}(pK_1 + pK_2)$. The *shape* of the profile will depend upon X, the ratio of the ionization constants (Eq. 10). The expected profiles for acids with $X = 4, 10, 10^2, 10^3$, and 10^4 are illustrated in Fig. 4.

The profile for a diprotic acid with $X = 4$ is identical with that of a monoprotic acid, and it is doubtful whether the profile for $X = 10$ could be experimentally distinguished from that of a monoprotic acid.

As X increases, the slope decreases; when X equals 100, the experimentally measured ionization interval would be expected to lie in the range of 4.1-4.7 pH units. When X is 10^3 or greater, the pro-
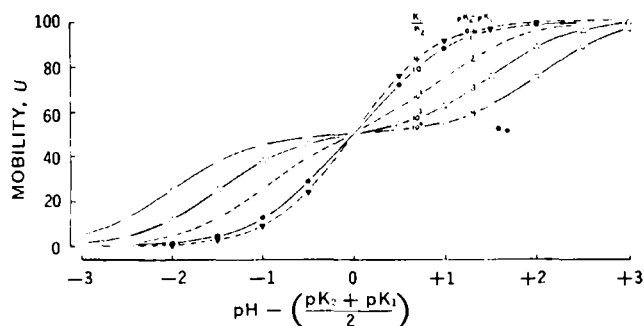
Figure 4—*Mobility-pH profiles of dicarboxylic acids.*

$$pH - \left(\frac{pK_2 + pK_1}{2}\right)$$

file would be expected to exhibit two sigmoid portions and the ionization interval for each of these portions would be expected to lie in the range of 1.9–2.4 pH units, identical to the value expected for a monoprotic acid. The ionization intervals for the profiles with X of 10^3 or more are measured with respect to the intercepts with the regions of 0 and 50 or 50 and 100 mobility units, since ionization of the individual functional groups is discernible. Thus, the mobility pH profile should serve to distinguish diprotic substances, when the pKa values of the ionizable groups differ by more than 1.5 or 2.0 units, from monoprotic substances.

Similar reasoning may be applied to determine the expected profiles for substances with three or more ionizable groups.

This theoretical description of electrophoresis strictly applies only to the migration of ions in free solution. Because of the tortuous path the migrating ions must follow around fibers and through constricted pores of the paper matrix, the rates of migration measured in paper electrophoresis are less than the mobilities measured in free solution. In addition, the phenomenon of electroosmotic flow and the variability of numerous parameters, including temperature, electrolyte viscosity and ionic strength, paper wetness, voltage gradient, and duration of a run, lead to considerable variability in measurements of migration rates.

However, if one prepares buffers of identical ionic strength and uses an electrically neutral substance to correct for electroosmotic flow and a completely ionized substance as a mobility standard, the effects of these variables cancel out in relation to the standard and reproducible values of relative mobility are obtained.

EXPERIMENTAL

Apparatus—A double-platen high-voltage paper electrophoresis apparatus[2] was used in conjunction with a power supply[3] (5000 v., 300 ma.) and a constant-temperature circulator[4]. The electrophoresis apparatus, which contains an upper and lower cooling platen, was modified (Fig. 5) by drilling and tapping the ends of the platens to permit attachment of aluminum angles [1.9 × 1.9 cm. (0.75 × 0.75 in.)] faced with a 0.63-cm. (0.25-in.) thickness of foam rubber.

Procedure—A sheet of Whatman 3MM filter paper [13.9 × 42.58 cm. (5.5 × 20.25 in.)] was marked at its midpoint with a light pencil line, and a series of light pencil lines were spaced at 5-cm. (2-in.) intervals on either side of the midpoint (Fig. 6).
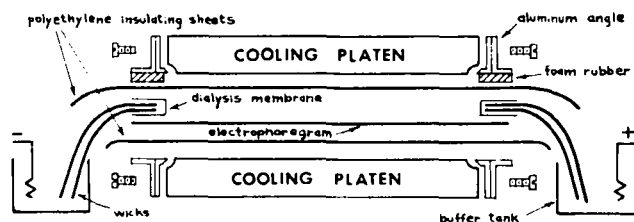

Figure 5—*Schematic diagram of electrophoresis apparatus.*
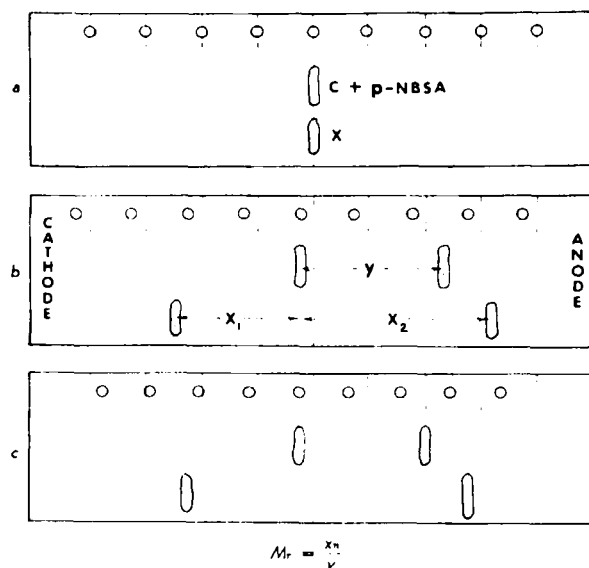

$$M_r = \frac{x_n}{y}$$

Figure 6—*Application of sample (x) and caffeine (c) and p-nitrobenzenesulfonic acid (p-NBSA) standards, evaluation of electrophoregram, and calculation of relative mobility (M_r). Key: a, before run; b, after normal run; and c, pattern distorted by electrolyte uptake via wicks.*

The paper was soaked in buffer for about 1 min., and some surplus buffer was removed by stroking each side of the paper with a glass rod. The wet sheet was centered on an 8-mil polyethylene [17.78 × 58.42 cm.(7 × 23 in.)] insulating sheet in the electrophoresis apparatus in which the circulating coolant had been adjusted to the desired temperature. A piece of "PE" polyester monofilament screen cloth[5] was placed on top of the paper, followed by three thicknesses of Whatman No. 1 filter paper and a second polyethylene insulating sheet. The apparatus was closed and a pressure of 5 psig. was applied to the lower platen for 2 min. When treated in this way at 20°, the Whatman 3MM paper reaches an equilibrium water content of approximately 71 % in 1 min. and no additional water is lost for as long as 5 min.

The apparatus was then depressurized and opened. Except for the midpoint, the exposed upper portion of the paper strip was covered with a polyethylene sheet to prevent evaporation. Approximately 10-μl. aliquots of samples were applied to the midline as streaks approximately 2 cm. long. A 10-μl. aliquot of an aqueous solution containing 0.2% caffeine and 0.2% p-nitrobenzenesulfonic acid was applied as one sample. The protective polyethylene was removed, and samples of a few microliters of a 0.2% caffeine solution were applied to each pencil line approximately 2.54 cm. (1 in.) from the upper edge of the paper (Fig. 6a).

Wicks were prepared from two thicknesses of 10.89 × 13.97-cm. (4.25 × 5.5-in.) Whatman 3MM paper soaked in buffer, and one narrow end was covered to a distance of 4.44 cm. (1.75 in.) from the end with a single thickness of dialysis membrane, obtained by slitting a piece of dialysis tubing, 4.44-cm. (1.75-in.) flat width[6]. The wicks were placed with the dialysis membranes in contact with either end of the electrophoregram and not extending onto the platen beyond the foam rubber pads. The upper polyethylene insulating sheet was placed in position, and the apparatus was closed and pressurized to 5 psig.

Two pieces of polyethylene tubing [1.27-cm. (0.5-in.) o.d. × 0.16-cm. (0.069-in.) wall × 5.08-cm. (2-in.) length], slit lengthwise, were placed as clamps over the sides of the polyethylene insulating sheets and extended slightly beyond the aluminum angles (Fig. 7) on the high tension end of the apparatus. The high tension is the anode with the power supply used[3]. Previous work showed that almost all electrical shorts occur at this end of the apparatus and that most of them result from the deposition of moisture between the insulating sheets; when the moisture reaches the edge of the sheet, a spark jumps to

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Table I—Composition of Buffers Used for Electrophoresis

Components A	B	Concentrations, mole/l. A	B	Approximate pH
Phosphoric acid	Monobasic potassium phosphate	0.090	0.050	2.0
Oxalic acid	Potassium hydroxide	0.071	0.050	2.0
Phosphoric acid	Monobasic potassium phosphate	0.008	0.050	3.0
Acetic acid	Potassium acetate	0.500	0.050	3.7
Acetic acid	Potassium acetate	0.050	0.050	4.7
Acetic acid	Potassium acetate	0.005	0.050	5.7
Monobasic potassium phosphate	Dibasic potassium phosphate	0.038	0.005	6.0
Monobasic potassium phosphate	Dibasic potassium phosphate	0.012	0.013	7.0
Monobasic potassium phosphate	Dibasic potassium phosphate	0.003	0.014	7.6
Triethanolamine	Hydrochloric acid	0.100	0.050	7.8
Triethanolamine	Hydrochloric acid	0.290	0.050	8.5
Ethylenediamine	Hydrochloric acid	0.055	0.050	9.0
Ethylenediamine	Hydrochloric acid	0.100	0.050	10.0
Piperidine	Hydrochloric acid	0.100	0.050	11.2
Piperidine	Hydrochloric acid	0.550	0.050	12.2
Potassium hydroxide	Potassium chloride	0.010	0.040	12.0

the grounded platen. Placement of the polyethylene clamps as indicated prevents the moisture from reaching the edge of the strip and virtually eliminates shorting.

The voltage was typically adjusted to 5 kv. and allowed to remain for 15 min. The apparatus was then opened, and the electrophoregram was kept in a horizontal position and examined while wet with a short wavelength (254 nm.) UV lamp[7]. Positions of the standards and any samples which were visible were outlined with pencil. The strip was then suspended vertically, with one longer side at the top, and dried for 10 min. at 90° in a chromatogram drying oven[8] with the draft control half open. The dry electrophoregram was again examined under UV light to check the uniformity of drying. Uneven evaporation of the water will be indicated by a shift of the standard spots, particularly caffeine, from the positions marked before drying.

The pattern after a normal run is indicated in Fig. 6b, where all caffeine spots moved a uniform distance of several millimeters toward the cathode. The pattern shown in Fig. 6c is distorted as a result of buffer uptake through the wicks and is typically observed when a dialysis membrane is not used.

Distances (X) to the centers of the various sample spots are measured from the center of the caffeine spot and divided by the distance (Y) from the center of the caffeine spot to the center of the p-nitrobenzenesulfonic acid spot, and this fraction is multiplied by 100 to give the relative mobility.

Radioactive Substances—The locations of radioactive spots were determined by cutting the dry electrophoregram into strips and scanning these in a radiochromatogram scanner[9]. This presented no problem when scanning for ^{14}C-labeled compounds with a polyethylene terephthalate[10] window covering the counter tube. However, scanning ^{3}H-labeled compounds with the counter in the windowless mode caused very erratic counter behavior, charac-

terized by spurious peaks and excessive count rates which drove the pen off-scale for prolonged periods. This behavior was traced to the attachment of conductive fibers of buffer-impregnated paper to the counter anode. This was overcome by modifying the counter to permit its operation with the strip feed and takeup reels rotating in a horizontal plane and the counter anode wires oriented vertically. Such operation required a counter gas flow rate of 400 ml./min. Careful attention must also be paid to the anode voltage, which should be no higher than the midpoint of the plateau region. These modifications totally eliminated the erratic counting with all buffers used except one composed of phosphoric acid and monobasic potassium phosphate at pH 2. Fibers from paper containing this buffer were readily attracted to the anode by electrostatic force, thereby preventing its use when scanning in the windowless mode.

Buffers—Buffers were prepared with an ionic strength of 0.05, using the components indicated in Table I. The pH was measured prior to each run using a pH meter[11] on the standard scale. After a run, the buffer in the anode and cathode tanks was remixed and saved for reuse.

RESULTS AND DISCUSSION

The contribution of this technique to the elucidation of the structures of certain drug metabolites will be illustrated by the following presentation of the mobility–pH profiles of several samples obtained synthetically or from the urine or bile of animals dosed with the parent drug. The profiles were obtained from measurements in buffers about 1–2 pH units apart over approximately the 2.5–11.5 pH range. The profiles are not intended to define the characteristics such as pKa or molecular weight of the substance
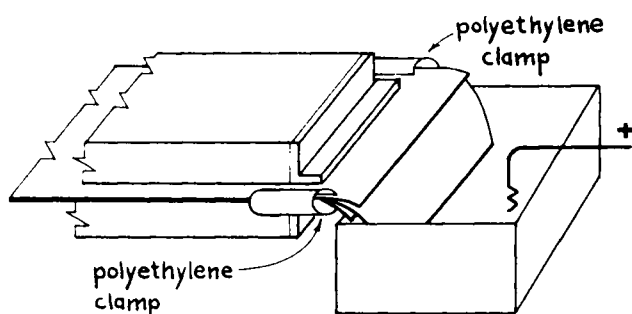

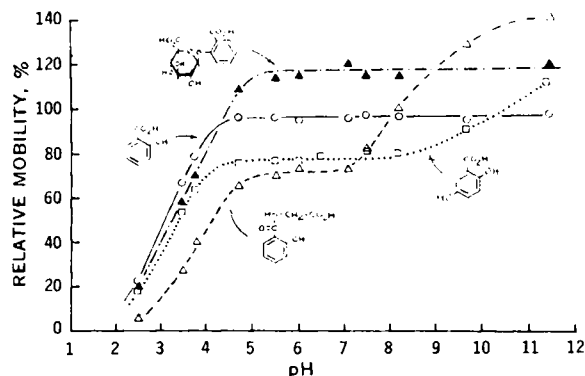Figure 7—*Placement of polyethylene clamps to eliminate shorting.*

Figure 8—*Mobility–pH profiles for salicylic acid (O), gentisic acid (□), salicyluric acid (△), and salicylic acid phenolic glucuronide (▲).*

[7] Mineralight, model UVS-12, Ultraviolet Products, Inc.
[8] National Appliance model 5670.
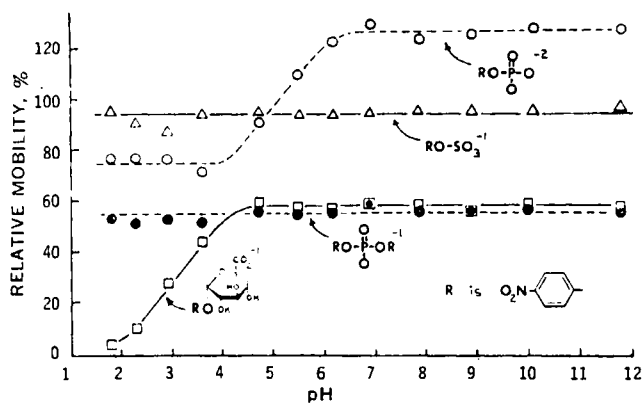[9] Packard model 7200.
[10] Mylar.
[11] Beckman Expandomatic.

Figure 9—*Mobility-pH profiles for* p-*nitrophenol sulfuric acid ester* (△), p-*nitrophenol glucuronide* (□), p-*nitrophenol dihydrogen phosphate* (○), *and* p-*nitrophenol monohydrogen phosphate* (●).

Figure 11—*Mobility-pH profiles for metabolites* (△,□,○) *found in urine following intraperitoneal administration of 1-naphthol-*[14]*C and profiles for authentic samples of conjugates of 1-naphthol* (*solid lines*) *redrawn from Fig. 10.*

with high precision, but they are sufficiently accurate to reveal characteristic structural features.

Salicylic Acid and Its Metabolites—The mobility-pH profiles (Fig. 8) of these substances are typical of those observed for weak acids, phenols, and glucuronide conjugates of acidic substances. They were obtained using synthetic samples of salicylic acid phenolic glucuronide, salicylic acid, gentisic acid, and salicyluric acid. The general shapes of the profiles are consistent with the properties discussed for Fig. 1. All of the substances show a pKa in the region of 3–3.5, characteristic of a carboxylic acid. Salicyluric acid shows a second pKa in the region of pH 9, consistent with ionization of a phenolic group. The mobility, 141, of salicyluric acid at pH 11.5 is approximately double that, 72, at pH 6, indicating the presence of two electrical charges and a single charge, respectively. Gentisic acid exhibits incomplete ionization of even one phenolic group at pH 11.5. Salicylic acid shows no ionization of the phenolic group at pH 11.5, as would be expected since the reported pKa for this group is 13.8 (23).

Closer scrutiny of the profiles reveals some deviations from the simple theory presented earlier. All of the substances migrate with relative mobilities less than those calculated, assuming mobility to be inversely related to the two-thirds power of the molecular weight. Predicted mobilities in the region of pH 6, relative to that of p-nitrobenzenesulfonic acid as 100, are: salicylic acid phenolic glucuronide, 150; salicylic acid, 130; gentisic acid, 120; and salicyluric acid, 106; the observed mobilities are 118, 96, 78, and 71, respectively. Thus, the predicted rank order of migration rates is observed, but the actual mobilities are considerably less relative to p-nitrobenzenesulfonic acid than predicted.

An ionization interval of 2.1 pH units was observed for salicylic and gentisic acids and 2.0 units for salicyluric acid. These are within the expected range of 1.9–2.4 pH units for monoprotic acids. The ionization interval of 3.0 units observed for the phenolic glucuronide
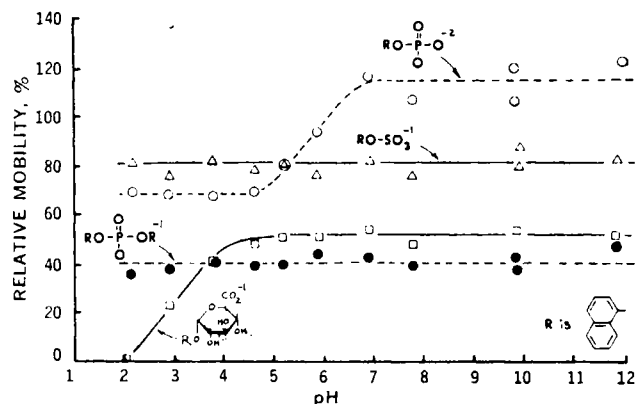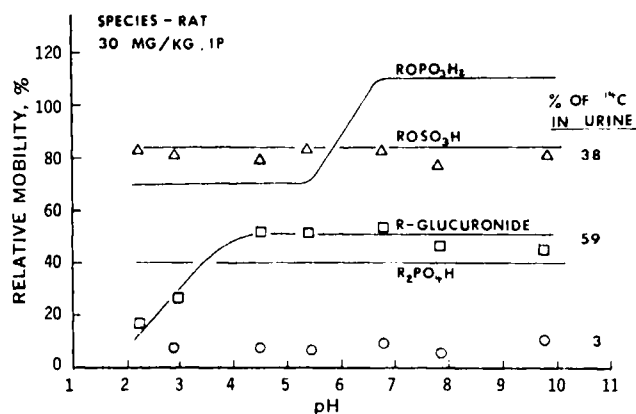
of salicylic acid is within the range expected for dicarboxylic acids, with ionization constants differing by 1–2 pH units.

Conjugates of p-Nitrophenol—These profiles (Fig. 9) illustrate the application of the technique to glucuronic, sulfuric, and phosphoric acid conjugates, for which electrophoresis is particularly appropriate. These highly polar, water-soluble compounds are not readily extracted with organic solvents and are, therefore, not easily studied by conventional TLC or paper chromatography.

The mobility-pH profiles of commercially available samples of the glucuronide, sulfuric acid ester, and mono- and dihydrogen phosphate esters of p-nitrophenol are shown in Fig. 9. Although the phosphate esters are not ordinarily found as drug metabolites in mammals, they are found in insects; it is, therefore, interesting to determine whether the mobility-pH profile will distinguish these conjugates.

The profile for the glucuronide shows virtually no mobility at pH 2, an ionization interval of 2.3 units with a pKa slightly over 3, and a region of constant mobility above pH 5. The sulfuric acid ester is fully ionized over the entire pH range and shows the expected constant mobility. A similar constant mobility is seen for the monohydrogen phosphate, but its mobility is much less than that of the sulfate, reflecting its higher molecular weight. The dihydrogen phosphate ester exhibits mobility at low pH, reflecting the low pKa of one of its protons, an ionization interval of 2.2 units with a pKa in the vicinity of 5, and constant mobility above pH 7 where the molecule bears two negative charges.

The mobilities are in the expected order based on molecular weights, except that one would not expect the considerable separation seen for the sulfate and dihydrogen phosphate at low pH since these two compounds are actually identical in molecular weight. In experiments using paper chromatography with the various buffers as the developing solvents, the sulfuric acid ester was retarded to a greater extent than the phosphate, suggesting that electro-

Figure 10—*Mobility-pH profiles for 1-naphthol sulfuric acid ester* (△), *1-naphthol glucuronide* (□), *1-naphthol dihydrogen phosphate* (○), *and 1-naphthol monohydrogen phosphate* (●).

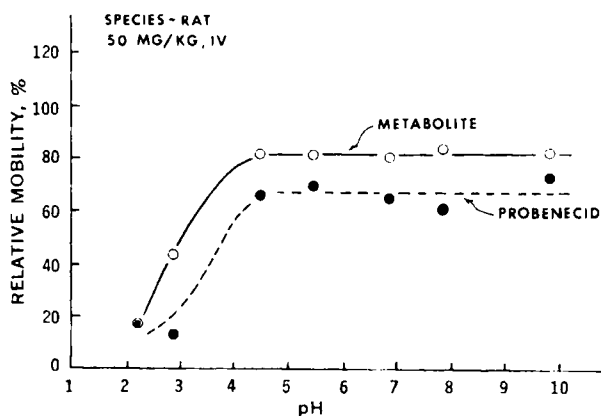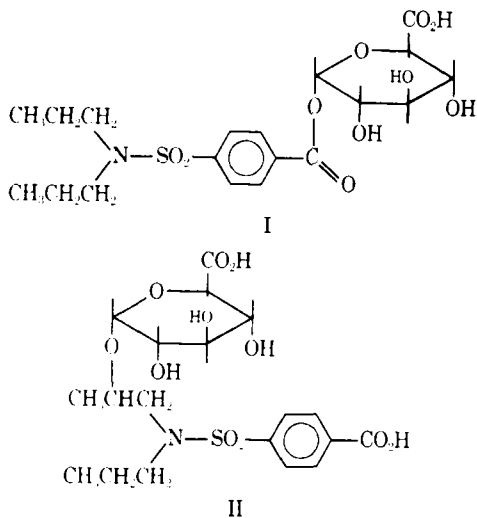Figure 12—*Mobility-pH profiles for probenecid* (●) *and a major metabolite* (○) *excreted in the bile of rats.*

I

II

phoretic separation of the two compounds is not a result of differential adsorption on the paper.

The mobility of the dihydrogen phosphate at high pH is not quite double that observed at low pH, as would be predicted by the presence of two electrical charges on the ion at high pH. A ratio of 1.7 was actually observed. Part of this discrepancy can be attributed to the fact that the runs were made in electrolytes of ionic strength 0.05. The theoretical ratio of 2.0 would be expected only at infinite dilution.

Conjugates of 2-Naphthol—The mobility-pH profiles of conjugates of 2-naphthol with glucuronic, sulfuric, and phosphoric acids are presented in Fig. 10. The profiles are analogous to the corresponding conjugates of p-nitrophenol and the same comments apply.

The applicability of this approach to the characterization of drug metabolites in a biological fluid was demonstrated after administration to a male rat by intraperitoneal injection of a 30-mg./kg. dose of 1-naphthol-^{14}C containing 10 μc. of ^{14}C. The mobility-pH profiles obtained using 10-μl. samples of the 24-hr. postmedication urine are shown by the data points in Fig. 11. The urine contained three substances. One moved with constant mobility over the entire pH range and accounted for 38% of the radioactivity. A second substance, accounting for 59% of the radioactivity, showed a pKa of about 3 and constant mobility above pH 4.5. The third material, which accounted for 3% of the radioactivity, remained at the origin.

This information alone suggests that the two major metabolites are conjugates with sulfuric and glucuronic acids. They are the metabolites of 1-naphthol in the rat. The lines in the figure were drawn independently from authentic samples of the indicated compounds and confirm the identity of the radioactive metabolites. The absence of either phosphate is apparent.

Metabolite of Probenecid—The mobility pH profile (Fig. 12) of a major metabolite excreted in rat bile after intravenous administration of probenecid-^{14}C was determined in conjunction with studies on the structure of the metabolite. It illustrates the value of the technique for detecting a metabolite of unexpected structure. Comparison of the metabolite profile with that of probenecid strongly suggested that the metabolite was not the expected acyl glucuronide of probenecid (I).

The molecular weight of I is 461, and this structure still contains only one ionizable group. Assuming the mobilities to be inversely related to the two-thirds power of the molecular weight, I would be expected to migrate about 60% as fast as probenecid, which has a molecular weight of 285. However, hydroxylation of probenecid followed by conjugation to an ether glucuronide would produce a species with a molecular weight of 477 and two carboxyl groups. The fully ionized molecule would then be expected to migrate about 27% faster than probenecid. The measurements in Fig. 12 indicate a 21% faster mobility for the metabolite. While it was not possible solely on the basis of this evidence to characterize fully the metabolite, it did guide further studies leading to the synthesis of p-(N-propyl,N-2-hydroxypropylsulfamoyl)benzoic acid, the aglycone of Metabolite II (21, 22).

Nitro- and Aminocatechols and Guaiacols—These profiles illustrate the application of the technique to compounds of similar
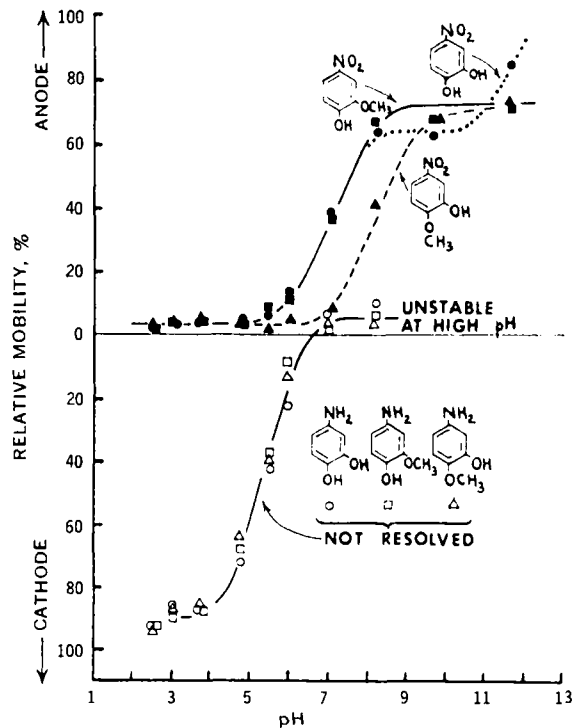

Figure 13 —Mobility-pH profiles for 4-nitrocatechol (●), 4-nitroguaiacol (■), 5-nitroguaiacol (▲), 4-aminocatechol (○), 4-aminoguaiacol (□), and 5-aminoguaiacol (△).

molecular weight that differ only slightly in pKa and to basic compounds. The five compounds which might result from O-methylation and reduction of the nitro group of 4-nitrocatechol were prepared by standard synthetic procedures in conjunction with a study of the metabolic fate of 4-nitrocatechol. The mobility-pH profiles of these substances and 4-nitrocatechol itself are shown in Fig. 13. The amino compounds were not resolved and, unfortunately, were chemically unstable in solutions above pH 8. However, their profiles in the acidic range were of the expected shape and exhibited pKa values in the range of 5.5. The phenolic compounds show no mobility below pH 5 where they are not ionized. Both 4-nitrocatechol and 4-nitroguaiacol are unresolved below pH 7.5 and exhibit pKa values of about 7. The two compounds are resolved above pH 8, where the mobility of nitrocatechol is somewhat less than would be predicted by its smaller molecular weight. Partial ionization of the second phenolic group in 4-nitrocatechol is seen at pH 11. The 5-nitroguaiacol, by virtue of the lesser acidity of the meta-nitrophenolic group, is separated from the other phenols in the range of pH 6-9. The observed pKa values for 4-nitroguaiacol and 5-nitroguaiacol are in the range of those reported for p-nitrophenol, pKa 7.15, and m-nitrophenol, 8.40 (23).
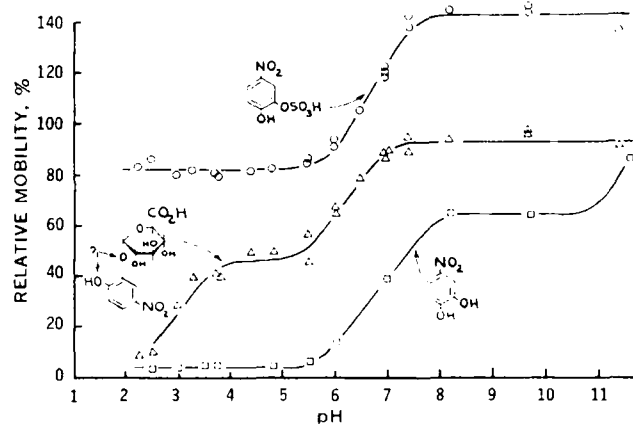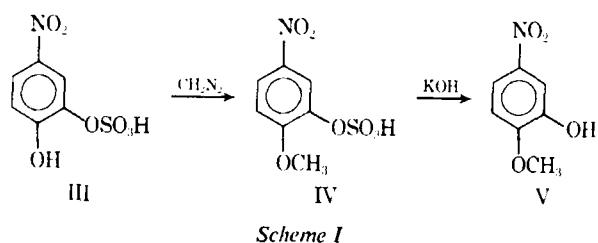

Figure 14 Mobility-pH profiles of metabolites excreted in the urine of rats given 30 mg./kg. of 4-nitrocatechol intraperitoneally.

Scheme I

Metabolites of 4-Nitrocatechol

Metabolites of 4-Nitrocatechol This study illustrates the use of electrophoresis to establish the formation of both sulfuric acid and glucuronic acid conjugates and then to establish further the position of sulfuric acid conjugation by chemical conversion of the conjugate to a derivative of known structure which could be unequivocally identified by its mobility-pH profile.

Two yellow spots could be detected in the 0–24-hr. urine of rats given 4-nitrocatechol (30 mg./kg. i.p.). Only traces of the parent compound were detected. The mobility-pH profiles of the metabolites and 4-nitrocatechol are shown in Fig. 14. The profile of the metabolite with highest mobility is characteristic of a sulfuric acid ester, and it is identical with the profile of an authentic sample of 2-hydroxy-5-nitrophenyl sodium sulfate (III, Scheme I). The observed pKa in the neighborhood of 7 is also characteristic of a *p*-nitrophenolic group. Unfortunately, the isomeric 2-hydroxy-4-nitrophenol is unknown, so that it was impossible to determine whether the isomeric sulfuric acid esters could be separated. To confirm further the structure of the metabolite, the residue from evaporation of a small aliquot of urine was extracted with methanol, the extract was treated with diazomethane, and the product was hydrolyzed with dilute potassium hydroxide. Only 5-nitroguaiacol (V) could be found when the hydrolysate was examined by electrophoresis. This establishes the metabolite as III since it has been shown (Fig. 13) that the isomers 4-nitroguaiacol and 5-nitroguaiacol (V) are separable by electrophoresis.

The other metabolite exhibits a profile characteristic of a conjugate with glucuronic acid and has pKa values in the ranges of 3 and 6.5. The pKa in the range of 6.5 appears characteristic of a *p*-nitrophenolic group and suggests that conjugation here also occurs *meta* to the nitro group.

The ratio of the mobility of the doubly charged glucuronic acid conjugate (92 units) to that of the singly charged species (46 units in the 4 5 pH range) is 2.0, in contrast to the ratio of 1.7 observed for the sulfuric acid ester, III, and for the dihydrogen phosphates presented earlier (Figs. 9 and 10). The deviation may result partly because of the proximity of the two charges in the dihydrogen phosphates and III.

Visual examination of the electrophoregrams suggest that about equal amounts of the glucuronide and sulfuric acid ester are formed.

Metabolites of Phenol Red—In this study a completely unexpected metabolite was detected, which was then shown to be derived from an impurity in the sample of dye. Phenol red itself and three metabolites were detected by their color and fluorescence on electrophoregrams of the urine and bile obtained from rats after intravenous administration of phenol red (15 mg./kg.). The mobility-pH profiles are presented in Fig. 15. The metabolite with the highest mobility (△) was excreted only in urine and exhibited a mobility-pH
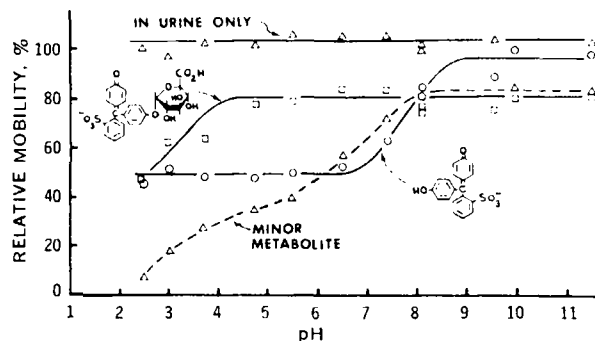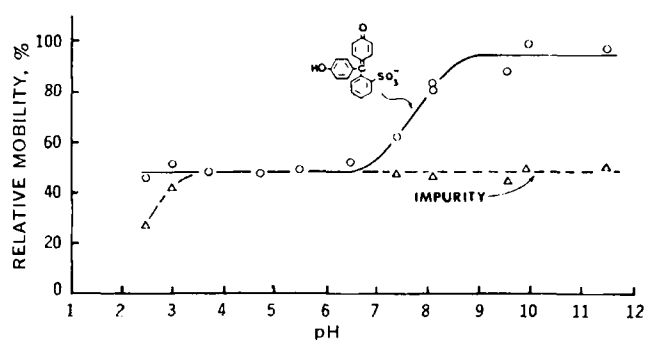

Figure 16 Mobility-pH profile of phenol red (O) and an impurity (△) which lacks the sulfonic acid group.

profile characteristic of a conjugate with sulfuric acid. A second metabolite (□), with a profile characteristic of a conjugate with glucuronic acid, was excreted in both urine and bile. The biliary excretion of the glucuronide of phenol red was reported previously (24). These two metabolites, as well as phenol red, exhibit significant mobility at low pH due to the ionized sulfonic acid group. However, a minor metabolite (△) was also detected; it lacked the sulfonic acid group, as indicated by its lack of mobility at pH 2.5.

It was supposed that this third metabolite might have arisen from a nonsulfonic acid impurity in the sample of phenol red. Careful examination of electrophoregrams of the particular sample of phenol red did, in fact, reveal the presence of an impurity (Fig. 16) that lacked the sulfonic acid function. When a pure sample of phenol red, in which this substance was undetectable, was administered to rats, the third metabolite was not excreted, confirming the supposition that it arose from the impurity.

SUMMARY

Characterization of ionizable substances by means of their mobility-pH profile was applied to organic substances by Franc and Kovar (25), who calculated mobilities in various buffers relative to the mobility in a standard buffer and plotted what they called an electrophoretic spectrum. Others (27 32) have also referred mobilities to standard substances including *p*-nitrobenzenesulfonic acid and have corrected for electroosmotic flow using neutral substances including caffeine. However, previous workers have not prepared buffers to constant ionic strength.

Control of ionic strength, combined with the use of *p*-nitrobenzenesulfonic acid as a reference substance and caffeine to correct for electroosmotic flow, forms the basis for obtaining reproducible relative mobilities over a wide pH range. The agreement of the mobility-pH profile thus measured with that predicted from elementary theoretical considerations is adequate to permit practical use of the technique for elucidating some structural features of simple organic molecules. Use of the method has been illustrated by applying it to the characterization of the metabolites of several compounds.

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Figure 15 —Mobility-pH profiles of phenol red (O) and its metabolites detected in the urine and bile of a rat given a 15-mg./kg. i.v. dose.

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Radioimmunoassay for Lysergide (LSD) in Illicit Drugs and Biological Fluids

LARRY J. LOEFFLER*▲ and JACK V. PIERCE†

Abstract ☐ A simple and convenient radioimmunoassay system was developed for the qualitative detection and quantitative determination of lysergide (LSD) in illicit drugs, human plasma, serum, or urine. The method utilizes a commercially available tritiated lysergide and specific antiserum, which can be obtained in large quantities from sheep after immunization with a lysergic acid-human serum albumin conjugate. Quantities of lysergide as low as 1 ng./ml. can be determined in plasma, serum, or urine, and lower levels can be detected qualitatively. The sensitivity of the assay is limited by the specific activity of the commercially available tritiated compound. Antibody cross-reactivity and, thus, interference in the assay were observed with other highly similar ergot alkaloids, such as ergonovine, methylergonovine, and ergotamine, but not with simpler indole structures.

Keyphrases ☐ Lysergide—radioimmunoassay of illicit drugs and biological fluids ☐ LSD—radioimmunoassay of illicit drugs and biological fluids ☐ Radioimmunoassay—lysergide (LSD) in illicit drugs and biological fluids ☐ Hallucinogens—radioimmunoassay of lysergide (LSD) in illicit drugs and biological fluids

The qualitative detection and quantitative determination of lysergide (LSD) in illicit drugs and in biological fluids have been a major interest in recent years because of the medical and social implications attendant its use. Chemical, spectral, and chromatographic methods for the determination of lysergide (IV*b*, Scheme I) were reviewed previously (1, 2), and it has been generally agreed that certain fundamental advances in micro-

analysis are required to detect and determine with confidence the nanogram or picogram levels, for example, of IV*b* or metabolites fleetingly present in urine or plasma after ingestion of the drug (1). A radioimmunoassay system (3) for IV*b* appears promising in this regard and was reported by one research group (4). This method (4) utilized rabbit antiserum to a lysergic acid–polylysine–hemocyanin conjugate, along with a specially synthesized lysergic acid–amino acid copolymer containing ^{125}I, and a double-antibody technique for the separation of free and bound IV*b* in the assay. Although details have not yet been reported, a commercial kit appears to be based upon similar methodology[1].

This paper reports the development and evaluation of a workable radioimmunoassay system for IV*b*. The system promises to be useful in the assay of illicit drug products or samples of biological fluids. The assay utilizes a commercially available tritiated IV*b* and specific antiserum which was obtained in large amounts from sheep after immunization with a direct covalent conjugate of lysergic acid (I) and human serum albumin (II). Separation of free and bound forms of IV*b* is achieved by the adsorption of unbound drug on dex-

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[1] Information supplied with kit from Collaborative Research Inc., Waltham, MA 02154; A. Taunton-Rigby, S. E. Sher, and P. R. Kelley, "Radioimmunoassay for D-Lysergic Acid Diethylamide."