Absorptiometric, Polarographic, and Gas Chromatographic Assays for the Determination of *N*-1-substituted Nitroimidazoles in Blood and Urine

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Abstract ☐ This report describes an absorptiometric, a polarographic, and a gas-liquid chromatographic assay procedure for the quantitative determination of an *N*-1-substituted 2- and 5-nitroimidazole in blood and urine. Using the same selective extraction procedure and thin-layer chromatographic separation of the ethyl acetate extracts of blood and urine, the compounds are eluted from the silica gel into methanol. A suitable aliquot of this methanolic extract can be assayed sequentially using the absorptiometric (Bratton-Marshall) assay which has a sensitivity limit of 0.5–1.0 mcg./ml., or the pulsed polarographic assay (sensitivity limit 0.2–0.3 mcg./ml.) or the electron capture–GLC assay using the trimethyl silyl (TMS) derivatives of these compounds (sensitivity limit 0.01–0.02 mcg./ml.) for quantitation. Thus the sequential analysis of a specimen of blood or urine covers a sensitivity range of three orders of magnitude (*i.e.*, from 0.01 to 10 mcg./ml. of sample). The absorptiometric assay was used in the determination of blood level fall-off curves and urinary excretion of each compound in a dog following the administration of single 10-mg./kg. doses by oral and i.v. routes, while the GLC assay was used to extend the limits of detection of the absorptiometric assay and to determine the blood level fall-off curve in man following a single oral 50-mg. dose of Compound I.

Keyphrases ☐ Nitroimidazoles, *N*-1-substituted—determination in blood, urine ☐ Blood, urine analysis—*N*-1-substituted nitroimidazoles ☐ Polarography—analysis ☐ GLC—analysis ☐ TLC—analysis ☐ UV spectrophotometry—analysis

The nitroimidazole class of compounds has yielded several antimicrobial agents that are currently in use as trichomonacides (metronidazole)[1] and amebicides (dimetridazole).[2] The above agents are 5-nitroimidazoles with antimicrobial activity. The synthesis of 2-nitroimidazole by Beaman *et al.* (1) and by Lancini and Lazzari (2) represents the first total chemical synthesis of this antibiotic agent hitherto isolated from a microbial strain resembling *Nocardia mesenterica*. This led to the synthesis of several *N*-1-substituted 2- and 5-nitroimidazoles which were screened for antimicrobial properties.

From among these 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol (hereafter referred to as Compound I) was synthesized by Beaman (3) and is under clinical investigation as a trichomonacidal agent, whereas α-chloromethyl-2-methyl-5-nitro-1-imidazoleethanol (hereafter referred to as Compound II) was synthesized by Hoffer (4) and is under clinical investigation as an amebicide. The chemical reactions of I and II are shown in Scheme I, and the analytical parameters used in the sequential analysis of the compounds by absorptiometric, polarographic, and gas chromatographic (GLC) methods are shown in Fig. 1.

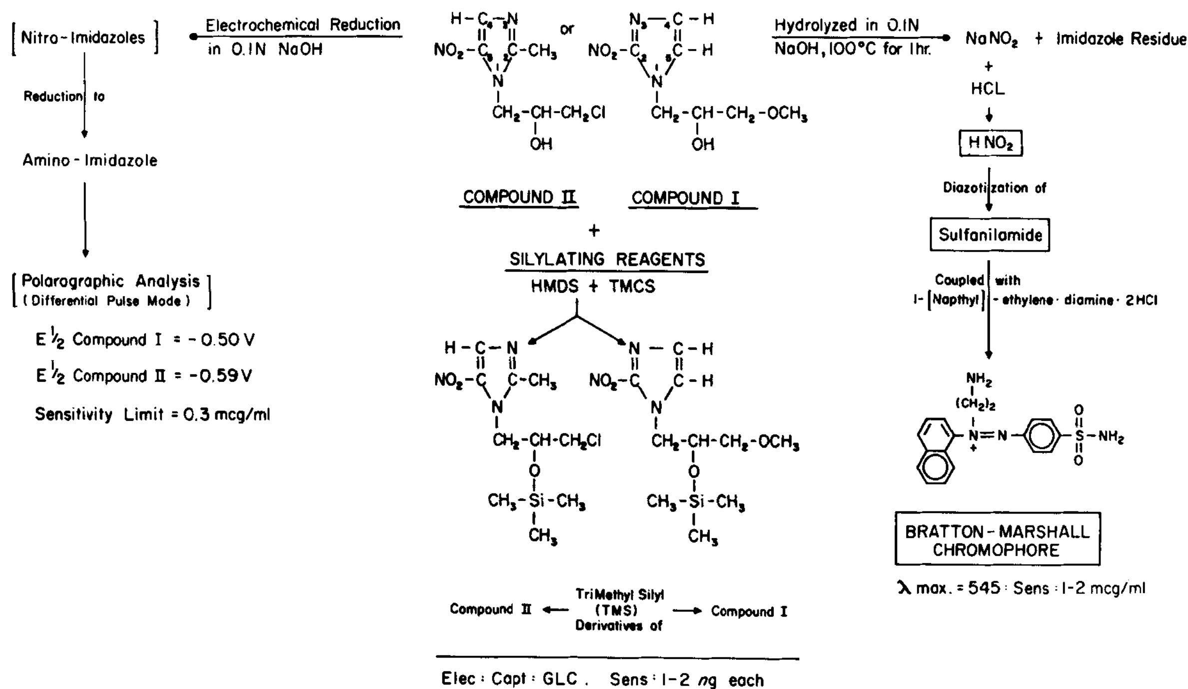
[1] 1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazole (Flagyl, G. D. Searle & Co).
[2] 1,2-Dimethyl-5-nitroimidazole (Emtryl).

The Ehrlich diazo coupling reaction between diazobenzene sulfonic acid and imidazole or phenol derivatives in acidic media to produce colored azo dye-chromophores has been widely used in biological chemistry for the detection of these physiologically important compounds. However, the reaction is inhibited by *N*-substitution or the presence of strong electronegative substituents such as the nitro group on the imidazole ring. Nitroimidazoles can be detected by the Bratton-Marshall method (5) following reduction of the nitro group to the corresponding amine. Stambaugh and Manthei (6) used this principle of reducing nitroimidazoles to their amino derivatives, followed by coupling with either diazotized sulfanillic acid, *p*-dimethylaminobenzaldehyde, or ninhydrin to produce a chromophore characteristic of the coupling agent used as a means of differentiating nitroimidazoles. These reactions were found to be unsatisfactory for use in solvent extracts of blood or urine due to the presence of many interfering substances of biological origin.

The most widely used analytical procedures for the analysis of nitroimidazoles in pharmaceutical or agricultural products has been by the use of polarographic techniques (7–10), which are sufficiently sensitive and specific but complicated because of the need of specialized instrumentation. The analytical parameters for the polarographic assay were developed by MacDonald *et al.* (11), and applied directly in the analysis of Compounds I and II in blood. The method was, however, not used for the analysis of biological specimens because of the superiority of the GLC assay.

A rapid and sensitive absorptiometric assay was reported by Lau *et al.* (12). It is based on the alkaline hydrolysis of *N*-1-substituted 5-nitroimidazoles to yield stoichiometric amounts of the nitrite (-NO_2) ion which after acidification is used to diazotize sulfanilamide and couple it to *N*-(1-naphthyl)ethylenediamine to produce the characteristic Bratton-Marshall azo dye chromophore (5). The authors found, however, that *N*-1-substituted 2-nitroimidazoles also undergo a similar reaction, and this principle was used to develop the absorptiometric assay described for the determination of both I and II in blood and urine with a sensitivity limit of 0.5–1.0 mcg. of compound/ml. of blood or urine and an overall recovery of about 70%. The TLC separation of the ethyl acetate extract was required to ensure the specificity of the assay.

The highly sensitive and specific polarographic and GLC assays described use the same extraction, thin-layer chromatographic separation, and methanolic elution steps described in the absorptiometric assay. Suitable aliquots of the methanolic solution are evaporated to dryness and analyzed sequentially by the polaro-



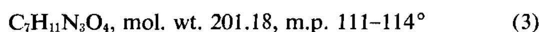
Scheme I

graphic and/or GLC assays as shown in Fig. 1. The GLC assay requires that both I and II be reacted with the silylating reagents at room temperature for 15 min. to produce their respective trimethyl silyl (TMS) derivatives (Scheme I). These derivatives are very sensitive to detection by electron-capture GLC and can be quantitated in the nanogram (10^{-9} g.) to picogram (10^{-12} g.) range. The GLC method has an overall recovery of 83% and a sensitivity limit of 0.01–0.02 mcg. of Compound I/ml. of blood. The method was applied to the determination of a blood level fall-off curve of Compound I in man, following a single oral 50-mg. dose and in extending the limits of detection of the absorptiometric assay.

EXPERIMENTAL

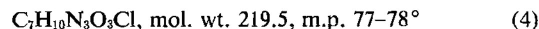
Absorptiometric Assay in Blood and Urine

Standard Solutions of Compounds I and II—*Compound I*—



$C_7H_{11}N_3O_4$, mol. wt. 201.18, m.p. 111–114° (3)

Compound II—



$C_7H_{10}N_3O_3Cl$, mol. wt. 219.5, m.p. 77–78° (4)

A. Stock Solution—Dissolve 10 mg. of Compound I in 100 ml. of methanol (100 mcg./ml.) in an actinic flask, store refrigerated, and make fresh weekly.

B. Working Solution—Dilute 1 ml. of A to 10.0 ml. with methanol (concentration 10 mcg./ml.). Make fresh daily. Aliquots of this solution (B) are evaporated to dryness and the residues are redissolved in blood and used as internal standards. Stock solution A′ and working solution B′ for Compound II are also prepared as described above.

Reagents—All reagents used were of analytical reagent grade purity (>98%) and all inorganic reagents were made up in distilled deionized water (d.d.w.).

1.0 *M* (pH 9.0) H_3BO_3–Na_2CO_3–KCl buffer solution: dissolve 62.8 g. of orthoboric acid (H_3BO_3) and 74.6 g. of KCl/l. of distilled deionized water. Dissolve 106 g. of Na_2CO_3/l. of distilled water. To 630 ml. of the boric acid–KCl solution, add 370 ml. of the Na_2CO_3 solution to make 1 l. of buffer solution. Shake well, check the pH, and adjust it to pH 9.0 if necessary with the Na_2CO_3 solution. The final solution is 1 *M* with respect to H_3BO_3–Na_2CO_3–KCl and should be stored at 37° to prevent the salts from crystallizing out of solution. The following are also used: methanol, absolute, reagent grade; NaCl, reagent grade crystalline salt; 0.1 *N* NaOH in distilled deionized water; and 4.0 *N* HCl in distilled deionized water. To make the 0.1% sulfanilamide solution, use *p*-aminobenzene-sulfonamide, mol. wt. 172.21, m.p. 163° (Matheson). Dissolve 100 mg. sulfanilamide in 5 ml. of glacial acetic acid and bring to 100 ml. with distilled deionized water. Bratton-Marshall coupling reagent *N*-(1-naphthyl)ethylenediamine·2HCl (Eastman), mol. wt. 259.25, m.p. 190°. Dissolve 100 mg. in 100 ml. of distilled deionized water. Ethyl acetate, reagent grade (Mallinckrodt) was the extracting solvent.

Procedure—Into a 40-ml. glass-stoppered centrifuge tube, add 2 ml. of blood or urine specimen, 4.0 ml. of pH 9.0 buffer solution, 5 g. of NaCl, and 15 ml. of ethyl acetate.

With each series of samples, run a 2.0-ml. specimen of control blood or urine (preferably taken from the subject prior to medication or from a pooled control source) and a set of internal standards consisting of 2.0-ml. specimens of control blood or urine added to 2.5, 5.0, and 10.0 mcg. each of Compound I or II [0.25, 0.50, and 1.0 ml. of the methanol working solution (B or B′), respectively, is evaporated to dryness under a stream of nitrogen at 35–40°]. Stopper the tubes (seal with a drop of distilled water), shake for 10 min. on a reciprocating shaker, centrifuge (0–5°) for 10 min. at 2000 r.p.m., and transfer the clear supernatant into a 15-ml. centrifuge tube. Repeat the extraction with another 10-ml. aliquot of ethyl acetate and centrifuge. While this step is in progress, evaporate the first ethyl acetate extraction in a 50° water bath under nitrogen to a small volume ≃ 1 ml. Combine the second extraction with the first and evaporate to dryness at 50° under nitrogen. Dissolve the residue in 100 μl. of ethyl acetate and transfer quantitatively onto a 20 × 20-cm. precoated (Silica Gel F_{254}) Brinkmann TLC plate. The TLC plate should be ruled into strips 2.54 cm. (1 in.) in width prior to the application of the samples. This is done to prevent the samples from diffusing into each other and also for a more uniform development of the chromatogram. Rinse the tube with 50 μl. of methanol and combine the wash on the TLC plate. External standards of 50 mcg. of Compounds I and II [0.5 ml. of stock solution (A or A′), respectively, are evaporated to dryness under nitrogen, the residue dissolved in 100 μl. of ethyl acetate, and transferred onto the TLC plate] are run alongside the sample extracts. The plate is developed in a vapor-saturated tank in chloroform–acetone–glacial acetic acid (85:10:7.5) until the solvent front has ascended 12–15 cm. The area on the TLC plate corresponding in R_f to that of the external standards of Compound I (R_f = 0.25–0.30) or Compound II (R_f = 0.35–0.40), respectively, is delineated using a sharp stylus.

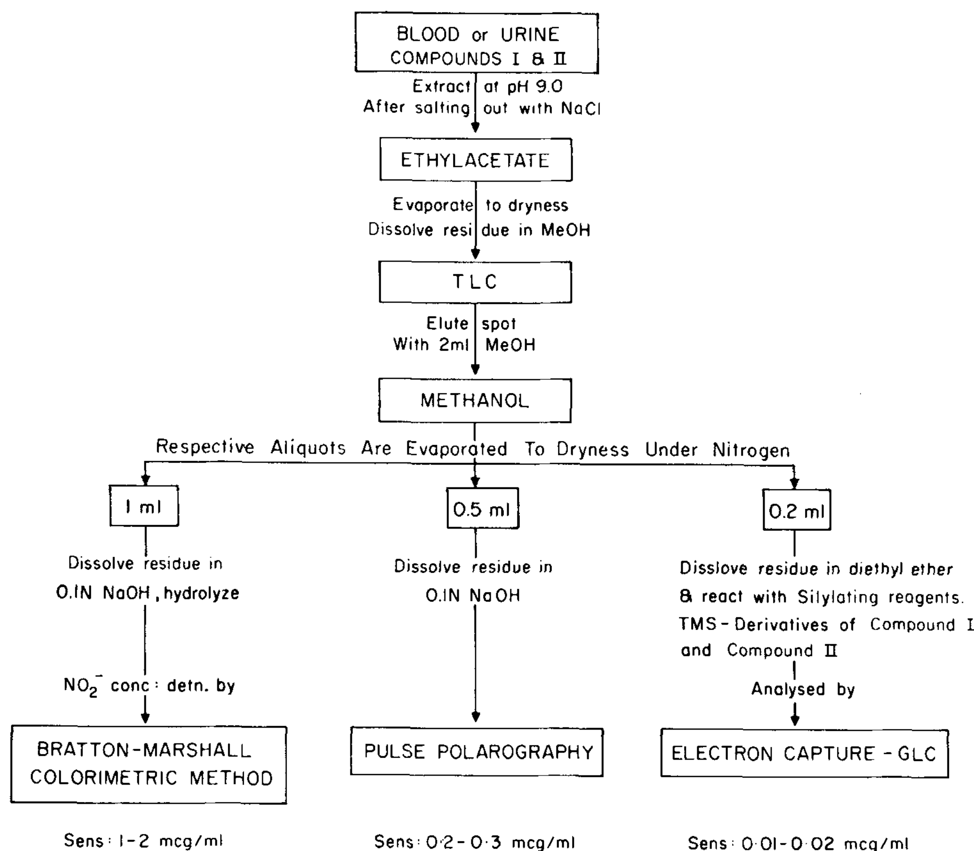


Figure 1—*Flow diagram of the sequential analysis of blood and urine for the determination of Compounds I and II.*

It must be noted that the visibly detectable limit of either Compound I or II on the TLC plate under shortwave UV is about 5–15 mcg. Amounts below this cannot be visibly defined; hence an area on the TLC plate 6.451 cm.^{2} (1 in.^{2}) corresponding to the R_f of the authentic standard is delineated, the silica gel is carefully scraped off, and transferred quantitatively into a 15-ml. centrifuge tube. The silica gel is eluted with 2 ml. of absolute methanol by slurrying for 1 min. on a vortex high-speed supermixer. The sample is centrifuged and the supernatant is decanted off carefully into another 15-ml. tube. The silica gel is reeluted with another 2-ml. aliquot of methanol as before, centrifuged, and the supernatants are combined. A 1.0-ml. aliquot of the methanolic solution is set aside for polarographic or GLC assay (if desired). The remaining supernatant ($\simeq$3.0 ml.) is evaporated to dryness under nitrogen in a 50° water bath and the residue is dissolved in 2.0 ml. of 0.1 *N* NaOH. However, if the sequential analysis of the sample using the polarographic and/or GLC assays is not required, then the entire sample is used for the absorptiometric assay.

At this stage external standards of Compound I or II in amounts of 2.5, 5.0, and 10.0 mcg. (0.25, 0.50, and 1.0 ml. of standard solution B or B′, respectively, are evaporated to dryness under nitrogen in a 50° water bath and the residues dissolved in 2.0 ml. of 0.1 *N* NaOH) are included for the determination of percent recovery and for establishing an analytical standard curve, Fig. 2A and B.

In the assay for Compound I *only*, transfer all the sample tubes and the external standards to a 100° water bath, allow to equilibrate for 5 min., stopper the tubes by sealing them with a drop of distilled water, and hydrolyze for 45 min. at 100°. In the assay for Compound II, however, all the sample tubes and external standards are hydrolyzed photolytically by irradiating them for 60 min. with UV energy from a Pyro Lux R-57 lamp (Luxor Corp., N. Y.). The samples are placed in a single row 30.48 cm. (12 in.) in front of the lamp which is housed in a aluminum foil-lined reflector box.

After hydrolysis, the samples are cooled in ice and acidified by adding 1.0 ml. of 4 *N* HCl. The samples are swirled on the vortex supermixer for 1 min. while cold, 0.5 ml. of 0.1% sulfanilamide solution is added, and the samples swirled again for 1 min. on the supermixer. Finally, 0.5 ml. of the coupling agent is added, the samples are swirled again on the supermixer for 1 min., and then allowed to stand at room temperature in the dark for 15 min. (minimum) to 30 min. (maximum) for full color development.

The absorbance of the Bratton-Marshall chromophore produced in each solution (final volume = 4.0 ml.) is measured in a 1-cm. path cell at 545 mμ in a Beckman DU or equivalent spectrophotometer.

Calculations—All biological samples are corrected for control (blank) values, and the external standards are corrected for reagent blank values.

Determination of Unknowns—The concentration of Compound I or II in the unknowns is determined by direct comparison of the absorbance of the unknowns to the mean absorptivity value of the three concentrations of the respective recovered internal standards thus:

$$\frac{A_{545}\ \text{unknowns}}{[a/\text{mcg./ml.}]\ \text{int. std.}} \times \frac{1}{\text{ml. of sample}} = \text{mcg. of Compound I or II/ml. of blood or urine}$$

Determine the A/mcg./ml. value of each of the three concentrations of recovered internal standards and calculate the mean A/mcg./ml. value of those standards. Use the mean value for determining the concentration in the unknowns.

Determination of Percent Recovery—The percent recovery of each internal standard is determined by direct comparison of the absorbance of the recovered internal standard against that of the corresponding external standards thus:

$$\frac{[A/\text{mcg./ml.}]\ \text{of int. std.}}{[A/\text{mcg./ml.}]\ \text{of ext. std.}} \times 100 = \text{percent recovery}$$

Polarographic Analysis

Procedure—The sample preparation was carried out as described for the absorptiometric assay up to the point where the compound is eluted off the silica gel successively with 2 × 2 ml. of methanol, the sample is centrifuged, and the supernatants are combined in a 15-ml. tube and a 1.0-ml. aliquot is set aside for polarographic or GLC assay (if desired). A 0.2-ml. aliquot of this sample is set aside for GLC analysis and the rest is evaporated to dryness under nitrogen. The residue is dissolved in 5 ml. of 0.1 *N* NaOH and then transferred into the polarographic cell. The three-electrode assembly is placed in the sample solution and the sample is deoxygenated with nitrogen for 2 min. The solution was analyzed in the differential pulse

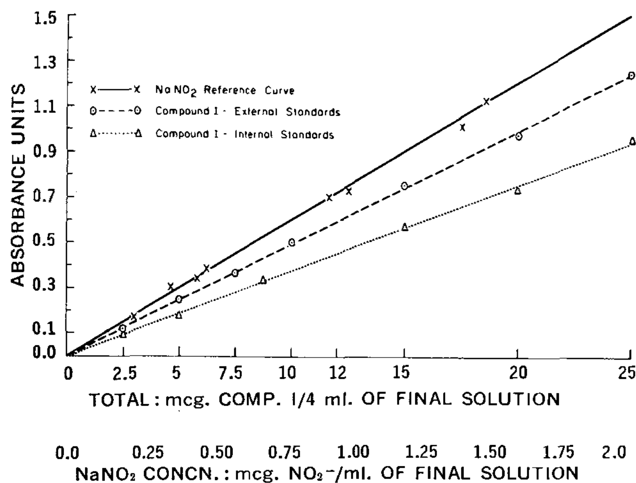


Figure 2A—*Standard curves of Bratton-Marshall chromophores of nitrite ion released by the alkaline hydrolysis of Compound I (external standards and internal standards recovered from blood including TLC) compared against an equivalent $NaNO_2$ reference curve.*

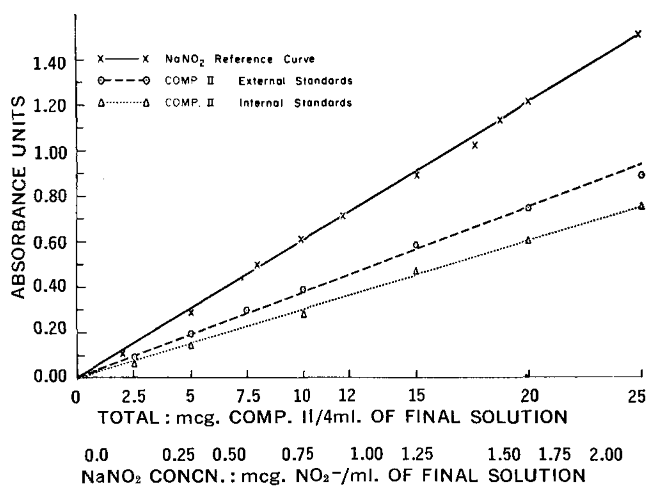


Figure 2B—*Standard curves of Bratton-Marshall chromophores of nitrite ion released by the photolysis in alkali of Compound II (external standards and internal standards recovered from blood including TLC) compared against an equivalent $NaNO_2$ reference curve.*

mode using instrumental parameters which will be published elsewhere (11).

The halfwave potential $E_{1/2} = -0.50$ v. for the nitro group reduction of Compound I and -0.59 v. for the nitro group reduction of Compound II, respectively. The peak area was calculated using height × width at half-height (cm.) and the sensitivity limit was 0.30 mcg. of I or II/ml. of final solution. A typical polarogram of the reference standard *versus* that of an internal standard of 5 mcg. each of Compound I or II recovered from blood is shown in Fig. 3.

Gas Chromatographic Assay in Blood and Urine

Principle—The GLC assay uses the same extraction procedure from blood or urine and the same TLC separation step as the absorptiometric assay. The silica gel covering a 6.45 cm.² (1 in.²) area corresponding in R_f to that of an external standard of Compound I or II is scraped off and eluted with methanol; the eluate is transferred to a fresh tube, evaporated to dryness, and the residue is vacuum dried. The residue is reacted with the silylating reagents to yield the trimethyl silyl (TMS) derivatives of I and II, respectively (Scheme I) and is quantitated by electron-capture GLC. The sensitivity limit of detection is of the order of 10–20 ng. of compound/ml. of blood or urine.

Reference Standard for GLC Assay—Since the assay involves several steps at which losses of compound are unavoidable, such as a TLC separation and a TMS derivatization step prior to quantitation, the use of a reference standard as a means of correcting for individual variations in recovery is good analytical practice. For this

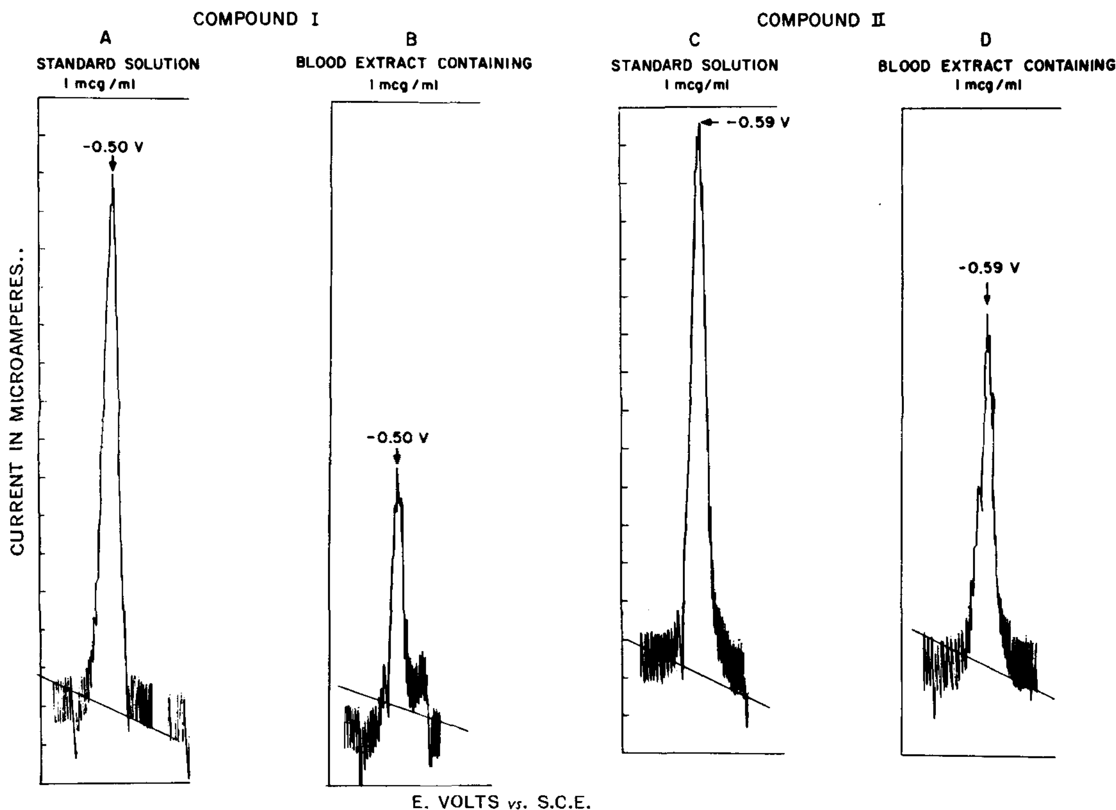


Figure 3—*Polarograms of standard solutions* (A *and* C) *and blood extracts* (B *and* D) *containing an added amount of Compounds I and II, respectively.*

purpose in the analysis of Compound I from biological media, an analogous nitroimidazole Compound II is used as the reference analytical standard and vice versa.

Standard Solutions—*Compound I*—Use working solution B from the absorptiometric assay (10 mcg./ml.). Suitable aliquots of solution B are evaporated to dryness and used as internal standards added to blood or urine for the determination of percent recovery. Aliquots of the same solution B are evaporated to dryness and used as external standards for determining the GLC standard curve.

Compound II Reference Standard—1-(3-Chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole, empirical formula $C_7H_{10}N_3O_3Cl$, mol. wt. 219.5, m.p. 77–78°. The stock solution (A′) and the working solution (B′) are made up as described for Compound I (see *Absorptiometric Assay*).

Parameters for GLC Analysis—*Column*—The column packing was a preconditioned phase containing 3% OV-17 on a 60/80 mesh Gas Chrom Q (Applied Sciences, State Park, Pa.) packed in a U-shaped 1.83-m. (6-ft.), 4-mm. i.d. borosilicate glass column.

Instrumental Parameters for GLC—A Micro-Tek gas chromatograph, model MT-220 (biomedical), was used equipped with a ^{63}Ni electron-capture detector containing a 10 mc. ^{63}Ni β-ionization source. Argon–methane (90:10) (oil pumped and dry) was used as the carrier gas, the column head pressure being adjusted to 40 psig. and the flow rate to 115–120 ml./min. The temperature settings were as follows: oven, 180°; injection port, 220°; detector, 290°. The conditions of column head pressure, flow rate, and oven temperature must be adjusted so as to obtain retention times of 5 and 6.5 min., respectively, for the TMS derivatives of Compounds I and II. A typical chromatogram is shown in Fig. 4. The solid-state electrometer (model No. 8169) input was set at 10^2 and the output attenuation was 16, giving a response of about 1.0×10^{-9} amp. for full-scale deflection (fsd); the chart speed was 76.2 cm./hr. (30 in.); and the time constant on the 1.0 mv. Honeywell recorder (model No. 194) was 1 sec. (fsd). The response of the ^{63}Ni electron-capture detector (operated in the pulsed d.c. mode) to the TMS derivatives of Compounds I and II showed maximum sensitivity at 30 v., at a 270-μsec. pulse rate and a 3-μsec. pulse width. Under these conditions a concentration of the TMS derivatives of 1 ng. of I:1.25 ng. of II gives nearly full-scale pen response on the 1.0-mv. recorder.

Calibration of Compound I by GLC—A calibration (external standard) curve of the peak area ratio of TMS-Compound I/TMS-Compound II *versus* concentration of Compound I/ml. of *n*-hexane (final solution) is constructed as shown in Fig. 5A. Determine the electron-capture detector response ratio of the TMS derivatives of Compounds I and II using the formula:

$$\frac{\text{response of Compound I}}{\text{response of Compound II}} = \frac{\text{peak area [height (cm.)} \times \text{width at half-height (cm.)] of Compound I}}{\text{peak area [height (cm.)} \times \text{width at half-height (cm.)] of Compound II}}$$

A fresh calibration curve is prepared for each day of analysis to establish the reproducibility of the GLC system. A similar curve of the added internal standards recovered from blood is also constructed. The amount of Compound I per aliquot of the unknown sample injected is calculated from the internal standard curve. In the analysis of Compound II, an external standard curve of the compound is constructed as shown in Fig. 5C using Compound I as the reference standard.

Since the peak area response ratio of Compound I:Compound II in any given sample is constant irrespective of the actual volume of sample injected, the concentration in the unknowns is obtained directly from the internal standard curve. Since the internal standards and unknowns are all dissolved in 1 ml. of *n*-hexane, there is no dilution or aliquot factor to be considered, unless the sequential analysis of the sample is employed (Fig. 1). The recovery factor for both internal and reference standards remains constant throughout and is not needed for the calculation of the unknowns.

Calculation of Unknowns—

$$\frac{\text{concn. (ng.) in unknown (extrapolated from int. std. curve)}}{\text{ml. of sample assayed}} \times \frac{\text{dilution factor}}{10^{-3}} = \text{mcg. compound/ml. of blood or urine}$$

where ng. compound = amount in the aliquot of sample injected extrapolated from the internal standard curve; dilution (aliquot) factor = sample aliquot (from sequential analysis if employed); and 10^{-3} = conversion factor for nanograms to micrograms.

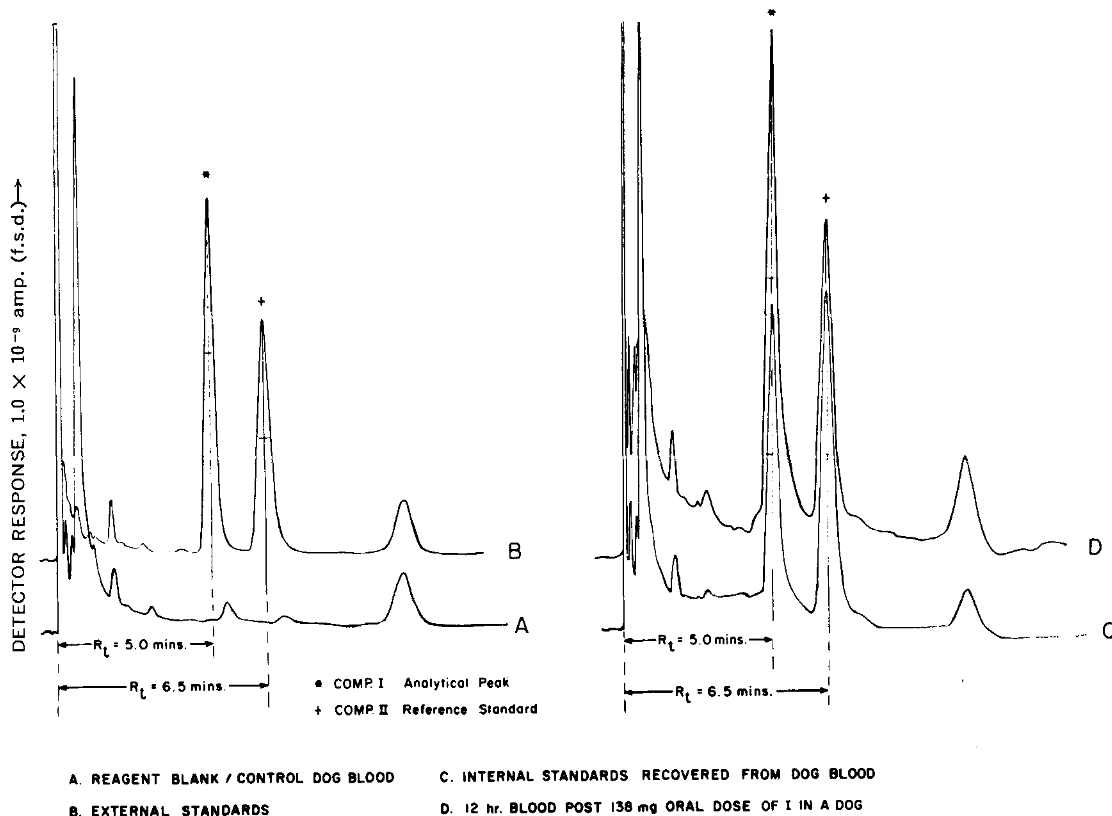


Figure 4—*Chromatograms showing the electron-capture GLC analysis of the TMS derivatives of:* (A *and* B) *the authentic standards of I and II and* (C *and* D) *Compounds I and II recovered from blood* in vitro *and* in vivo.

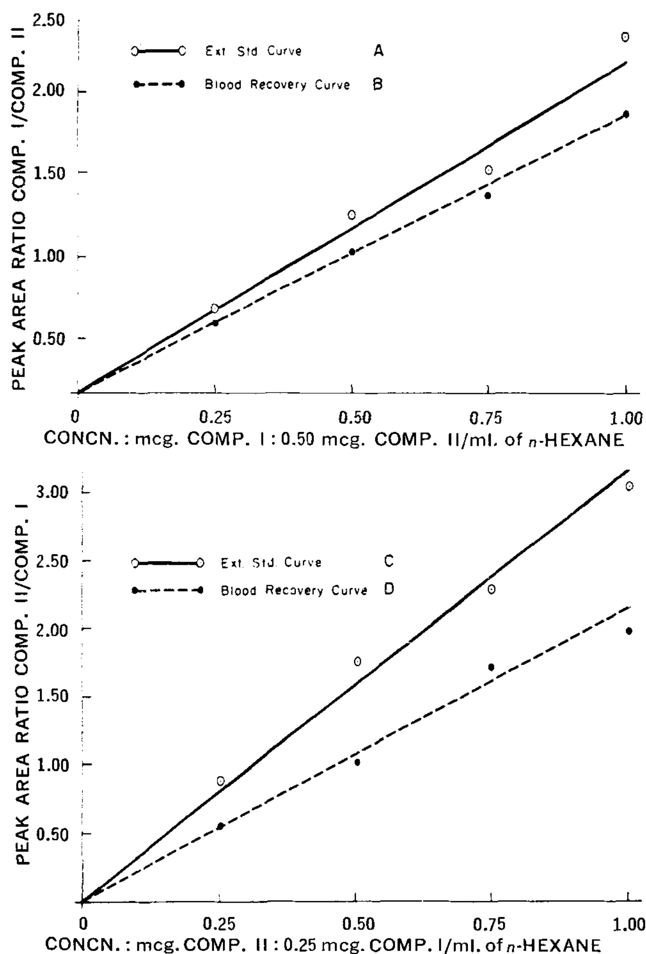


Figure 5—*Standard curves of the electron-capture detector response to the TMS derivatives of Compound I using Compound II as the reference standard* (A/B) *and vice versa* (C/D).

Sample Preparation for GLC Analysis of Blood and Urine—*Reagents*—Same as for the *Absorptiometric Assay*. In addition the following silylating reagents are required: hexamethyldisilazane (HMDS); trimethylchlorosilane (TMCS, Applied Science Labs, State College, Pa.); *n*-hexane, Fisher Spectrograde reagent (anhydrous).

Procedure for Blood and Urine—Into a 40-ml. glass-stoppered centrifuge tube, add 50 μl. of reference standard solution B′ of Compound II equivalent to 500 ng. and evaporate off the ethanol under nitrogen. Add a 2-ml. specimen of blood or urine and mix sample for 1 min. on a vortex high-speed mixer. Then add 4.0 ml. of pH 9.0 borate buffer, 5.0 g. of NaCl, and 15 ml. of ethyl acetate to the sample tube. Stopper the tube (sealing it with a drop of distilled water). Along with the unknowns, run a 2-ml. control specimen of blood or urine (taken prior to medication) and triplicate control specimen added to the residues of 250, 500, and 750 ng. of Compound I (25, 50, and 75 μl. of solution B) as the internal standard and 500 ng. of Compound II (50 μl. solution B′) as the reference standard. If the sequential analysis of the samples is used (Fig. 1), then 500 ng. of Compound II must be added to the aliquots taken of both internal standards and unknowns, the methanol evaporated to dryness, and the residues silylated as described. Follow the entire extraction procedure, TLC separation of the ethyl acetate residue, and methanol elution of the respective silica gel areas corresponding to Compound I (R_f = 0.3) and Compound II (R_f = 0.4) as described in the absorptiometric assay. However, in the GLC assay *only*, the silica gel areas of Compounds I and II are combined in a 15-ml. centrifuge tube prior to elution into methanol. The samples are then eluted successively with 2-ml. portions of methanol by slurrying for 60 sec. on a vortex high-speed supermixer. The samples are centrifuged, the clear supernatant transferred quantitatively (by decanting) into another 15-ml. centrifuge tube, evaporated to dryness at 50° under nitrogen, and vacuum-dried for 15 min. (under mild vacuum) to remove all traces of moisture.

At this point in the assay a series of external standard solutions of Compound I covering the concentration range of 100 to 750 ng. in suitable increments are prepared for the determination of the external standard curve. Suitable aliquots of standard solution B, Compound I, are pipeted into 15-ml. centrifuge tubes into which 50 μl. of solution B′ containing 500 ng. of Compound II (the reference standard) are added. The methanol is evaporated to dryness and vacuum-dried to remove all traces of moisture. These standards are processed along with the sample extracts to standardize the silylation process.

The residues are dissolved in 250 μl. of diethyl ether followed immediately by the successive addition of 40 μl. of HMDS and by 20 μl. of TMCS (silylating reagents) into the ether solutions. The tubes are stoppered immediately and mixed vigorously on a vortex supermixer for 60 sec. and then allowed to stand at room temperature (25°) for 15 min. to effect complete silylation of the two compounds. During this time period the samples are mixed again on the vortex mixer about 5–6 min. after the addition of the reagents. The sample tubes will develop a white precipitate of NH_4Cl due to the silylation reaction. This precipitate does not affect the reaction since the silylating reagents are present in a large excess relative to the amount of compound to be silylated.

After the 15-min. reaction period, the tubes are unstoppered and the solvent is evaporated to dryness under nitrogen at room temperature to remove the ether and all of the silylating reagents. The TMS derivatives are dissolved in 1 ml. of *n*-hexane and suitable aliquots of these standards are injected into the GLC instrument for preparing the external standard curve. This standard curve is required to establish the reproducibility of the GLC system and also for recovery determination. All the sample residues are also dissolved in 1 ml. of *n*-hexane (anhydrous) added to each tube, stoppered, and mixed well for 60 sec. on a vortex supermixer to dissolve the TMS derivatives. A suitable aliquot of this *n*-hexane extract (1–10 μl.) is injected into the gas chromatograph for the quantitation of Compound I. The large dilution is necessary because of the high sensitivity of the electron-capture detector to the TMS derivatives of these compounds and also to reduce the sample blank.

Determination of Percent Recovery—The use of a reference standard (Compound II) in the analysis, together with the internal standard (Compound I) added to blood or urine, eliminates the need to determine the percent recovery obtained with each run. Once the actual recovery factor has been experimentally determined by GLC (*viz.*, 83 ± 2.5% for Compound I and 65 ± 5.0% for Compound II), this factor can be assumed to be constant throughout. Since the relative recovery of each compound is assumed to remain constant, any change in the overall recovery of one would be automatically reflected in the recovery of the other to the same degree. Hence, irrespective of the actual recovery of each, the response ratio of the electron-capture detector would be constant and thus compensate for any variations incurred in sample processing. Consequently the response ratio of the TMS derivatives of Compound I/Compound II in the recovered internal standards and in the unknowns would reflect the relative amounts of each compound present irrespective of the actual recovery obtained.

If the determination of the actual recovery is desired, then proceed as follows: prepare a series of standards of either Compound I or II (100 to 1000 ng.) added to blood or urine as before but *without* the addition of the appropriate reference standard. Prepare the samples as described up to the silylation reaction step. At this point, add 250 ng. of Compound I or 500 ng. of Compound II (reference standard) to the appropriate series of samples and vacuum dry the residues. Continue with the silylation reaction as described previously, dissolving the final residue containing the respective TMS derivatives in 1 ml. of *n*-hexane. A suitable aliquot of this solution (1–10 μl.) is analyzed by GLC. The peak area response ratio of the recovered standards is directly compared against that of the external standards to determine the percent recovery of Compound I or II, respectively.

Calculation of Percent Recovery of Added Standards—The peak area response ratio of the recovered standards is plotted against concentration of the added standards of Compound I (Fig. 5B) to construct a blood-recovery curve. The slope of this curve is compared directly with that of the external standard curve of Compound I (Fig. 5A) to obtain the overall percent recovery. The same procedure is applied in the determination of the recovery of Compound II using Compound I as the reference standard. The slope of the blood-recovery curve of II (Fig. 5D) is compared directly with that

of the external standard curve (Fig. 5C) to determine the overall recovery. Alternatively, the response ratio of any one recovered standard is compared directly with that of the same concentration of the external standard, thus:

$$\frac{\text{[peak area response ratio] rec. std.}}{\text{[peak area response ratio] ext. std.}} \times 100 = \% \text{ recovery}$$

The sensitivity of the GLC method is of the order of 0.01–0.02 mcg. of either Compound I or Compound II/ml. of blood or urine, but the overall recovery from blood of Compound I is of the order of 83 ± 2.5%, whereas that for Compound II is 65 ± 5.0% (Table I).

RESULTS AND DISCUSSION

A systematic investigation of suitable analytical parameters for use in the quantitation of *N*-1-substituted nitroimidazoles was undertaken in the development of suitable analytical procedures.

The UV absorption spectra of the two compounds determined in 0.1 *N* HCl showed absorption maxima at 325 mμ (*A*/mcg./ml. = 0.038) for Compound I and at 278 mμ (*A*/mcg./ml. = 0.029) for Compound II, respectively. The specific absorptivity was sufficient to quantitate 10–100 mcg. of each compound/ml. of final solution. The determination of the extractability of these compounds into organic solvents from blood buffered at different pH values was done initially using the UV absorption of these compounds in 0.1 *N* HCl for quantitation. It was determined that using ethyl acetate as the extracting solvent gave better than 90% recovery of these nitroimidazoles from blood buffered to pH 9.0. The recovery declined significantly at pH values greater or less than 9.0.

The ethyl acetate extract was evaporated to dryness, the residue dissolved in 0.1 *N* HCl, and backwashed with diethyl ether as a cleanup step prior to quantitation in the UV. Not withstanding the cleanup of the sample, high UV absorption values from control blood suggested the incorporation of a TLC separation step for specificity and additional cleanup of the sample prior to quantitation. The sensitivity limits of the UV assay were unsatisfactory for use as a routine method due to high blank values from control blood and urine.

The principle of the sensitive absorptiometric assay reported by Lau *et al.* (12) was used to advantage in the absorptiometric assay described for the quantitation of these nitroimidazoles in blood and urine. The kinetics of the hydrolysis (at 100°) of Compound I to the -NO_2 ion indicated that the reaction was complete in 45 min., giving a >80% yield of nitrite ion. The yield was linear with concentration (2.5–25 mcg./4 ml.) measured against an external standard curve of $NaNO_2$ in concentrations equivalent to a theoretical 100% yield of -NO_2 from Compound I (Fig. 2A). Following diazotization of sulfanilamide with the released -NO_2 ions, coupling of the diazonium salt with *N*-(1-naphthyl) ethylenediamine · 2 HCl to form the characteristic Bratton-Marshall azo dye chromophore was complete in 30 min. The hydrolysis of Compound II (at 100°) to the -NO_2 ion however was erratic. Although the overall yield was >70% it was not very reproducible. The compound was noted to be unstable to light exposure, the investigation of which led to the finding that the compound undergoes photolysis to the -NO_2 ion in alkali when irradiated with UV energy from a Pyro Lux R-57 lamp. The kinetics of the photolysis of Compound II in alkali to yield -NO_2 indicated that the reaction was complete in 60 min., giving a >80% yield of nitrite ion. The yield was also linear with concentration (2.5–25 mcg./4 ml.) (Fig. 2B).

Due to the intense color of the chromophore and its high absorption, the useful range for quantitation was in the concentration range of 0.25–5.00 mcg./ml. of final solution, Fig. 2A and 2B, respectively. Concentrations of either compound greater than 5.0 mcg./ml. should be diluted.

Initial recovery experiments were conducted from blood buffered to pH 9.0, extracted into ethyl acetate, the residue of which was hydrolyzed directly in alkali and quantitated by the Bratton-Marshall procedure. The erratic recoveries and high blank values obtained indicated the need for extensive cleanup of the sample prior to quantitation. The incorporation of a TLC separation step to ensure specificity and sample cleanup, the use of deionized distilled water to reduce possible contamination with nitrite or nitrate ions in preparing the reagents, and the use of NaCl to salt out the lipoproteins after buffering the blood or urine to pH 9.0 with 1.0 *M* borate buffer achieved the necessary cleanup of the sample. The residues of the ethyl acetate extracts on TLC analysis were free of

Table I—Recovery of Compounds I and II from Blood and Urine Determined by the Electron-capture GLC Assay

ng. Compd. Added/2 ml.	ng. Compd. Recovered	% Recovery
A—Recovery of Compound I from Blood		
250	210	84.0
500	425	85.0
750	600	80.0
1000	845	84.5
Mean 83.4 ± 2.5		
B—Recovery of Compound II from Blood		
250	170	68.0
500	315	63.0
750	530	71.0
1000	600	60.0
Mean 65.5 ± 4.9		
C—Recovery of Compound II from Urine		
250	221	88.2
500	397	79.4
750	588	78.4
Mean 82.0 ± 5.4		

interfering substances, especially in the areas on the chromatoplate corresponding in R_f to that of Compounds I and II.

The final procedure yielded an overall recovery for Compounds I and II of 72 ± 5.7% and 70 ± 7.2% from blood, respectively (Table II, A and C). The recovery from blood was linear in the concentration range of 2.5–25 mcg./4 ml. of final solution (Fig. 2A and 2B). The recovery from urine was of the order of 59 and 53%, respectively (Table II, B and D). The sensitivity limit of the assay is 0.50–0.60 mcg. of Compound I or 1–2 mcg. of Compound II/ml. of blood or urine using a 2-ml. specimen per assay and a 2:1 sample–blank absorbance ratio as the limit of detectability.

The sensitivity limits of the absorptiometric assay can be extended into the submicrogram range using the polarographic assay and further extended into the nanogram range using the GLC assay. Therefore, if the sequential analysis of a specimen of blood or urine is undertaken as shown in Fig. 1, the ultimate sensitivity limit of quantitation obtained is of the order of 10–20 ng. of compound /ml. of blood or urine by electron-capture GLC. The polarographic assay was not investigated in any great detail since its usefulness in the analysis of nitroimidazoles in general has already been demonstrated (7–11). However the recovery of 5–15 mcg. of Compounds I and II was determined to be of the order of 47 and 60%, respectively, with a limit of detectability of 0.3 mcg. of compound/ml. of blood.

The relatively low melting point and the presence of the -NO_2 group in the imidazole ring of these compounds suggested that they could be readily quantitated by electron-capture GLC with potential sensitivities in the nanogram range. However, initial attempts at GLC analysis revealed very poor response characteristics with minimum detectability in the 1–5-mcg. range together with tailing peaks. It was apparent that the polar hydroxyl groups present in the side chain of the molecules (Scheme I) were interacting with the polar liquid phase to cause tailing and also possibly reduce the electron-capture response of the -NO_2 group due to inductive effects on the imidazole ring. The silylating of hydroxyl groups on aliphatic side chains and in sterically unhindered aromatic rings is known to proceed readily at room temperature (13). The preparation of suitable silyl derivatives of these compounds was effected using diethyl ether as the solvent and was found to proceed almost instantaneously at room temperature when reacted with hexamethyldisilazane (HMDS) synergized by trimethylchlorosilane (TMCS). The reaction products were analyzed by TLC using the solvent system heptane–$CHCl_3$–ethanol, 50:50:10 (v/v). The chromatoplate showed the presence of a single UV absorbing spot (R_f = 0.50) for the trimethyl silyl (TMS) derivatives of Compounds I and II. No residual I (R_f = 0.20) or II (R_f = 0.25) was seen on the plate, indicating complete reaction. The TMS derivatives when eluted off the silica gel with diethyl ether and analyzed by GLC gave excellent response to the electron-capture detector with nanogram range sensitivity with well-resolved peaks demonstrating the feasibility of the method. An aliquot of the *n*-hexane solution of the reaction mixtures when analyzed directly by GLC also gave a single sharp well-resolved peak with the same retention time and sensitivity as the respective TLC-

Table II—Recovery of Compounds I and II[a] from Blood and Urine Determined by the Bratton-Marshall Absorptiometric Assay

mcg. Added/ 2 ml. Blood	Internal Standard, E/mcg./ml.	External Standard, E/mcg./ml.	% Recovery
A—Recovery of Compound I from Blood			
2.5	0.158	0.195	81.0
5.0	0.169	0.246	68.7
5.0	0.134	0.205	65.2
5.0	0.139	0.200	69.5
7.5	0.123	0.198	63.0
10.0	0.174	0.236	73.7
10.0	0.168	0.231	72.7
10.0	0.122	0.176	69.3
10.0	0.118	0.176	67.0
10.0	0.110	0.176	63.0
15.0	0.151	0.201	75.0
15.0	0.144	0.201	71.6
20.0	0.139	0.176	79.0
20.0	0.137	0.172	79.7
20.0	0.146	0.194	75.3
25.0	0.152	0.198	76.8
Mean 71.91 ± 5.74			
B—Recovery of Compound I from Urine			
10.0	0.107	0.178	60.1
10.0	0.102	0.178	57.0
Mean 59.0 ± 2.3			
C—Recovery of Compound II from Blood			
2.5	0.100	0.174	58.0
2.5	0.104	0.174	59.8
5.0	0.116	0.174	66.7
5.0	0.114	0.174	65.8
10.0	0.132	0.166	79.5
10.0	0.124	0.166	74.7
10.0	0.118	0.164	72.2
10.0	0.082	0.128	64.1
10.0	0.083	0.128	64.8
10.0	0.096	0.136	70.6
20.0	0.137	0.166	82.5
20.0	0.101	0.140	72.1
20.0	0.102	0.140	73.1
Mean 69.53 ± 7.21			
D—Recovery of Compound II from Urine			
10.0	0.068	0.128	53.1
10.0	0.066	0.128	51.6
Mean 52.4 ± 2.4			

[a] Compound I is 1-(3-methoxy-2-hydroxypropyl)-2-nitroimidazole; Compound II is 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole.

isolated TMS derivatives, indicating that the reaction products were homogeneous and free from contaminating impurities. The silica gel area on the TLC plate corresponding in R_f to that of any unreacted residual parent compound that may have been present in the reaction mixture but undetectable by UV was eluted with methanol, evaporated to dryness, resilylated using the HMDS-TMCS mixture, and reanalyzed directly by electron-capture GLC. The chromatogram did not show any residual TMS derivatives, indicating that the initial silylation reaction was quantitative at room temperature.

Optimum conditions for the concomitant silylation of both compounds were obtained in diethyl ether as solvent using a 2:1 ratio of HMDS:TMCS and a total reaction time of 15 min. at room temperature for quantitative reaction. It is essential that the two silylating agents be added successively into the reaction mixture rather than from a synthetic mixture of the two reagents and that they also be vigorously mixed immediately after addition. The reaction mixture is also mixed vigorously at intervals of 5 and 10 min. after the addition of the reagents during the 15-min. reaction period. The reaction mixture is evaporated to dryness under nitrogen and the residue is dissolved immediately in anhydrous *n*-hexane without any vacuum drying. Although the TMS derivatives are stable in anhydrous *n*-hexane, they should nevertheless be analyzed by GLC as soon as possible. These derivatives are easily hydrolyzed by traces of moisture; hence it is of the utmost importance to maintain an anhydrous environment during their preparation and subsequent analysis by electron-capture GLC. The response of the electron-capture detector operated in the pulsed d.c. mode to the TMS derivatives of I and II is linear with concentration in the range of 0.1 to 1.0 mcg. of compound/ml. of *n*-hexane equivalent to 1.0 to 10 ng./10 µl. of sample injected. The sensitivity of the detector was such that 1.00 ng. of TMS-I and 1.25 ng. of TMS-II gave peak heights equivalent to nearly full-scale pen response for each compound on the recorder at the electrometer settings described. Since the response of the electron-capture detector to nearly equal (nanogram) amounts of the TMS derivatives of both compounds was comparable, *i.e.*,

$$\left[\frac{\text{peak area of TMS-I}}{\text{peak area of TMS-II}} \simeq \frac{1.0}{0.8} \simeq 1.25\right]$$

the peak area ratio of the two was used as the index of detector performance and overall efficiency of the analytical procedure. Thus the external standard curve and the blood- or urine-recovered internal standard curves were constructed by plotting the peak area response ratios of TMS-I/TMS-II *versus* concentration of I containing a constant amount of II as the reference standard/ml. of *n*-hexane or vice versa when Compound II is the analytical peak and Compound I the reference standard. The overall recovery of 250–1000 ng. of Compound I or II from blood was of the order of 83 ± 2.5% and 65 ± 5.0%, respectively (Table I, A and B), whereas that of Compound II from urine was 82 ± 5.4% (Table I, C). The GLC method was used to extend the range of sensitivity of detection of the absorptiometric assay in the determination of blood level fall-off curves in the dog and in man.

Application of the Absorptiometric Assay in Biological Specimens—The Bratton-Marshall absorptiometric assay was applied to the determination of blood and urine levels of I and II in a dog following the administration of a single 138-mg. dose of I and a 122-mg. dose of II (equivalent to 10 mg./kg.) by i.v. and oral routes, respectively.

The blood level fall-off curves of Compounds I and II are shown in Figs. 6 and 7, respectively. Following administration by both oral and i.v. routes, blood levels were measurable for up to 12 hr. by the absorptiometric assay, beyond which time period they were nonmeasurable by this method (*i.e.*, <0.5–1.0 mcg./ml.). Both compounds showed a typical biphasic i.v. blood level fall-off curve with a rapid initial distribution phase (half-life ≃ 20–30 min.) followed by a linear elimination phase with a half-life of 2.51 and 6.10 hr. for Compounds I and II, respectively.

The oral blood level fall-off curves of both compounds indicate good absorbability, Compound II showing a rapid absorption pattern with a blood level peak at 1 hr. of 11.5 mcg./ml. while Compound I showed a slower absorption pattern with a blood level peak at 2 hr. of 9.0 mcg./ml. Both compounds showed a linear elimination phase, Compound I having a faster elimination (half-life ≃ 2.55 hr.) running almost parallel with its i.v. elimination, while Compound II showed a slower elimination pattern (half-life ≃ 5.30 hr.). The blood levels in the 12–72-hr. postmedication period, where measurable, could only be determined by the electron-capture GLC assay with a sensitivity limit of the order of 0.01–0.02 mcg./ml. of blood.

The urinary excretion data following i.v. and oral administration of Compounds I and II are given in Table III, A and B, respectively. The data indicate that approximately equal amounts of both Compound I or II are recovered in the 0–72-hr. collection period following either i.v. or oral administration. This amounts to a recovery of 6.2 and 6.9% of Compound I and 7.1 and 6.6% of Compound II following i.v. and oral administration, respectively. The low percent recovery of the administered dose indicates either extensive biotransformation, tissue distribution, and/or alternate routes of elimination.

Application of the GLC Assay to Biological Samples—A blood level fall-off study on Compound I was conducted in a human subject following the administration of a single 50-mg. oral dose (0.67 mg./kg.). This study was primarily designed to test the capabilities of the GLC assay in the determination of blood levels following therapeutic doses.

The blood level fall-off curve, Fig. 8, indicates rapid absorption of the drug with a blood level peak of 1.0 mcg./ml. at 30 min. postdosing. The levels declined to 0.5 mcg./ml. at 1 hr. followed by a plateau in the levels up to 11.5 hr. Between 11.5 and 48 hr. the levels declined progressively, with a half-life of about 8 to 9 hr. Because of

Table III—Urinary Excretion Data on Compounds I and II in a Dog (Absorptiometric Assay)

Route	Time Period, hr.	mcg./ml.	Total mcg.	Cumulative Total mcg.	Excretion Rate, mcg./hr.	% of Dose Recovered
			A—Compound I[a]			
i.v.	0–23	34.0	8500	8500	370.0	6.2
	23–47	n.m.[b]	—	—	—	—
	47–71	n.m.	—	—	—	—
Oral	0–24	38.7	9288	9288	387.0	6.7
	24–48	1.0	340	9628	14.2	6.9
	48–72	n.m.	—	—	—	—
			B—Compound II[c]			
i.v.	0–2	1.10	340	340	170.0	0.3
	2–24	36.8	8290	8630	376.8	7.07
	24–48	1.10	80	8710	3.3	7.14
	48–72	n.m.[d]	—	—	—	—
Oral	0–24	18.1	4440	4440	185.0	3.64
	24–48	15.1	3650	8090	152.1	6.63
	48–72	n.m.	—	—	—	—

[a] Following single 138-mg. doses by i.v. and oral routes. [b] n.m. = not measurable, *i.e.*, <0.5–0.6 mcg. I/ml. urine using a 2-ml. specimen per assay. [c] Following single 122-mg. doses by i.v. and oral routes. [d] n.m. = not measurable, *i.e.*, <1.0 mcg. II/ml.

its inherent sensitivity the GLC assay thus can be used to determine blood levels in man up to 48 hr. postoral dosing following a single 50-mg. dose, and also in extending the limits of detection of the absorptiometric assay in determining blood levels in the nanogram (10^{-9} g.) range of sensitivity. This was demonstrated in the blood level fall-off curve of Compound II in a dog (Fig. 7) which indicates the range of detectability of the two assays.

Specificity of the Assay—Studies on the metabolism of the 5-nitroimidazole, metronidazole (14), have indicated that the $-NO_2$ group is stable to metabolic reduction *in vivo* and that metabolism occurs preferentially on the alkyl substituents resulting mostly in the excretion of the resulting alcohol, as such, and as the glucuronide and sulfate esters.

Since the $-NO_2$ group is left intact the presence of any metabolites in blood or urine would interfere with the specificity of the absorptiometric assay since they too would yield $-NO_2$ ion upon alkaline hydrolysis. The specificity of the three assays described for Compounds I and II is ensured by the inclusion of the TLC-separation step which effectively resolves the intact drug from other possible metabolites and from extracted impurities. Two-dimensional TLC examination of the silica gel eluates of the spots corresponding in R_f to the intact compound (I and II) migrate as one component when cochromatographed with the respective authentic standard. Thus the spot assayed following one-dimensional TLC represents the respective intact compound only. This was further verified using the GLC assay of the compounds as their respective TMS derivatives following elution from the silica gel.

No metabolites were seen in either the blood or urine specimens in a dog following the administration of Compound I. However, several metabolites were seen in blood and urine in a dog following the administration of Compound II. Since they were all well resolved from the intact compound by TLC analysis, they do not interfere with the specificity of the three assays for Compound II.

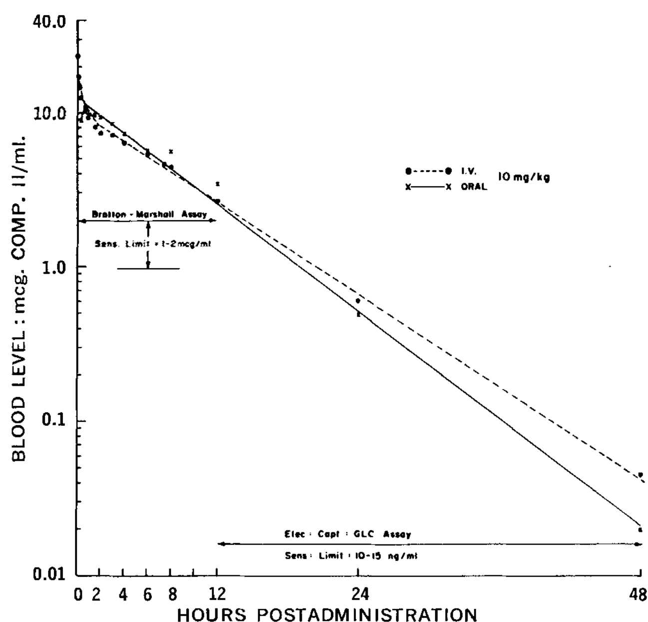


Figure 7—*Blood level fall-off curves of Compound II in a dog following the administration of a single 122-mg. dose by i.v. and oral routes.*

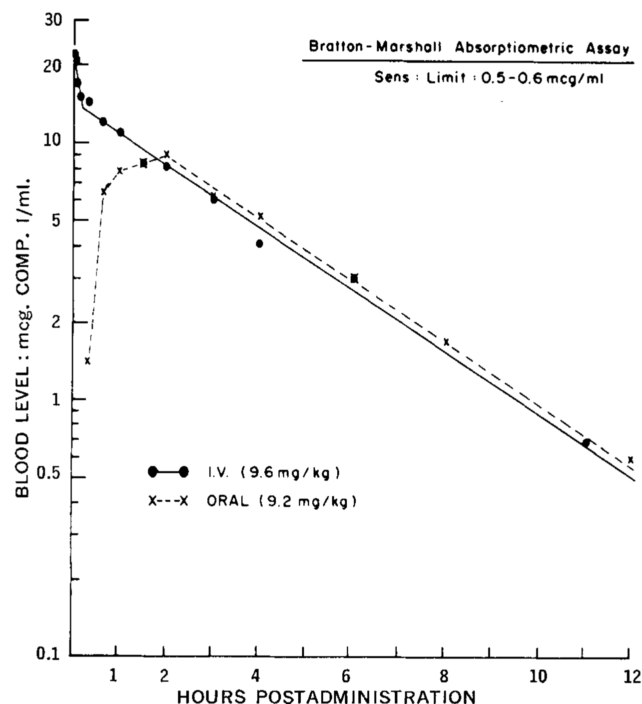


Figure 6—*Blood level fall-off curves of Compound I in a dog following the administration of a single 138-mg. dose by i.v. and oral routes.*

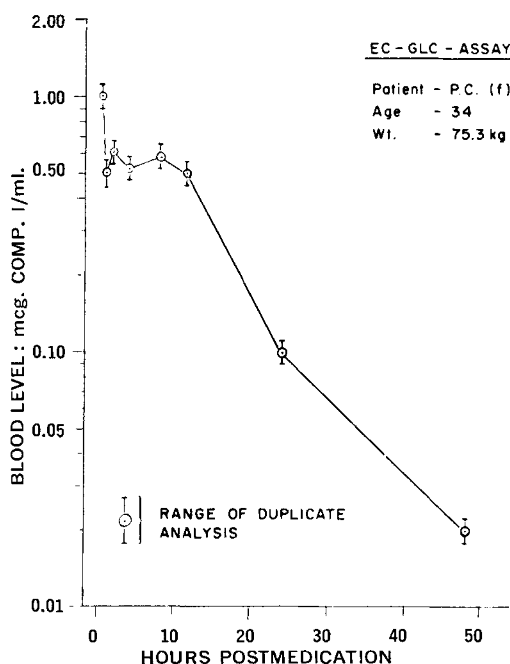


Figure 8—*Blood level fall-off curve of Compound I in man following the administration of a single oral 50-mg. dose.*

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Estimation of Mean Potency and Content Uniformity of Tablets: A New Approach

J. P. COMER, H. L. BREUNIG, D. E. BROADLICK, and C. B. SAMPSON

Abstract ☐ Current official procedures for the estimation of mean drug level and of content uniformity in individual tablets do not efficiently utilize all information available to the analyst, nor can confidence statements be made concerning the reported results. The authors present here a plan for combining readily available weight data with that from assays to generate distributions of potency per tablet. Tolerance limits on these distributions reflect not only the proportion of tablets bracketed, but also the degree of confidence to be placed in the finding. Reference is made to Monte Carlo studies on theoretical distributions as well as to examples from production lots of certain tablet items. The efficient utilization of this combined information leads to an improved method for estimating mean potency and content uniformity.

Keyphrases ☐ Tablets—mean potency, content uniformity ☐ Drug content uniformity, mean potency estimations—tablets ☐ Equations—tablet drug uniformity, mean potency ☐ Computer simulation—drug distribution, tablets

When considering drug dosage forms, the primary concern of the ethical pharmaceutical industry is that the patient receive in his individual dose an amount of medicament close to that claimed on the label. If this is so, the physician may prescribe the product with confidence that the desired drug substance will be available to perform its intended function. There are many facets to pharmaceutical quality assurance but all lead toward ensuring the identity, safety, and bioavailability of the drug of interest. This paper is solely concerned with the amount of drug substance in the unit dose. Such considerations as particle size, rates of dissolution and of absorption, freedom from impurities, and numerous others, while understood to be parts of pharmaceutical quality assurance, are not directly considered here.

Although this paper refers to compressed tablets, the techniques presented could also apply to filled capsules, ampuls, and other dry product dosage forms. Because of variation in homogeneity of granulation and in individual tablet weights, it is obviously unrealistic to expect every unit of product to possess *exactly* the same amount of physiologically active drug, but with good manufacturing practice these variations may be controlled. The subject of drug substance variability has been considered by a number of authors. Olson and Lee (1) have summarized much of the discussion and present an extensive list of references. A more recent paper is that by French *et al.* (2). Breunig (3) has emphasized the importance of weight control for individual units of product. Roberts (4) points out how easily many tablets (and filled capsules and sterile solids) may fail the USP weight variation test when based upon a sample of 20. He develops four rules for acceptance based upon the coefficient of variation of unit weights and provides charts which may be used for evaluation.

PRODUCT SPECIFICATIONS

The existence of variability in pharmaceutical products is recognized by USP XVII (5) and NF XII (6). These official compendia include at least three types of product specifications:

Rubric Limits—Referred to in the separate monographs, they are the bounds within which the mean response of samples of N units of product must fall based upon physiological, biological, or chemical assay. This response is in terms of the weight of drug substance per unit of product as determined upon individual units or as drug substance weight per average unit where test methods applicable to single units are not available. The bounds and the