Determination of Nitroimidazoles in Biological Fluids by Differential Pulse Polarography

M. A. BROOKS ˣ, L. D'ARCONTE, and J. A. F. de SILVA

Abstract □ A sensitive differential pulse polarographic assay was developed for the determination of metronidazole, ornidazole, and 2-nitro-1*H*-imidazole-1-(3-methoxy-2-propanol) in plasma. The compounds are selectively extracted into ethyl acetate from a protein-free filtrate of plasma, buffered to pH 7.0 ± 0.2. The residue of the ethyl acetate extract is dissolved in 0.1 *N* NaOH and analyzed by differential pulse polarography for the reduction of the nitro group at approximately −0.600 v *versus* the saturated calomel electrode. The overall recovery from plasma was about 55 ± 3.0% (*SD*) for the three compounds investigated. A TLC step after the ethyl acetate extraction may also be included to ensure specificity. This step reduced the overall recovery to approximately 45%. The sensitivity limit of detection from plasma using a 2-ml sample is 0.1 μg/ml. The assay may also be employed for the analysis of urine. The urine is adjusted to pH 7.0 ± 0.2 and extracted with ethyl acetate, and the residue is analyzed as described for plasma. The assay was applied to the determination of ornidazole in blood and urine in the dog following 10 mg/kg po.

Keyphrases □ Nitroimidazoles—polarographic analysis, biological fluids □ Metronidazole—polarographic analysis, biological fluids □ Ornidazole—polarographic analysis, biological fluids □ Polarography—analysis, nitroimidazoles, biological fluids

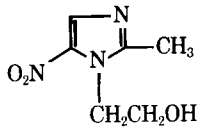
The nitroimidazoles are antimicrobial agents and have been determined in biological fluids by various assay procedures.

These procedures include the spectrophotometric determinations of metronidazole (2-methyl-5-nitro-1*H*-imidazole-1-ethanol, I) (1), ornidazole [2-methyl-5-nitro-1*H*-imidazole-1-(3-chloro-2-propanol), II] (2), and 2-nitro-1*H*-imidazole-1-(3-methoxy-2-propanol) (III) (2); the polarographic determinations of I (3, 4), II (2), III (2), dimetridazole (1,2-dimethyl-5-nitroimidazole, IV) (5), and ipronidazole (2-isopropyl-1-methyl-5-nitroimidazole, V) (6); the spectrodensitometric determination of I and tinidazole [1-[2-(ethylsulfonyl)ethyl]-2-methyl-5-nitroimidazole, VI] (7); and the GC determination of I (8), II (2), and III (2).

The present work is a modification of the assay of de Silva *et al.* (2) and enables the polarographic assay to be performed on a routine basis with sensitivities as low as 0.1 μg/ml in biological fluids. The assay differs from the previous work (2) by the use of a protein-free filtrate of plasma, which yields extracts that can be assayed without further cleanup.

The assay was employed for the determination of I–III in plasma and urine; the compound of interest is selectively extracted into ethyl acetate either from a protein-free filtrate of plasma or from urine buffered to pH 7.0 ± 0.2. The residue of the ethyl acetate extract is dissolved in 0.1 *N* NaOH and analyzed by differential pulse polarography. A TLC separation step may be included to ensure specificity.

Blood levels of II and the urinary excretion of the unchanged drug were determined in the dog following a 10-mg/kg dose.



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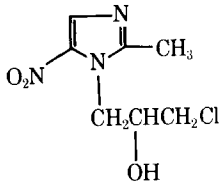

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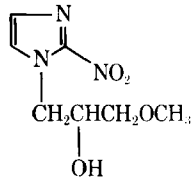

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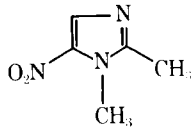

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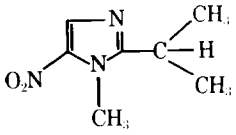

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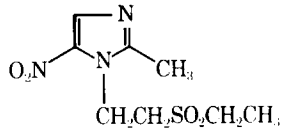

VI

EXPERIMENTAL

Conditions for Polarographic Analysis—A polarographic analyzer[1] equipped with a drop timer[2] was used in conjunction with a three-electrode polarographic cell comprised of a dropping mercury electrode, a saturated calomel electrode (SCE), and a platinum wire as the auxiliary electrode, as previously described (9). The drop time was 1.0 sec, and the drop mass was 2.32 mg/sec, where $(m^{2/3})(t^{1/6}) = 1.7525$. The current range was typically 0.5–5 μamp for a peak response of full-scale deflection, the scan range was 1.5 v, and the scan rate was 2 mv/sec.

The samples were scanned between −0.350 and −0.750 v *versus* the SCE, and the polarograms were recorded on an *X–Y* recorder[3].

[1] PAR model 174, Princeton Applied Research Corp., Princeton, N.J.
[2] PAR model 172A, Princeton Applied Research Corp., Princeton, N.J.
[3] Houston Omnigraph model 2200-3-3, Houston Instruments, Bellair, Tex.

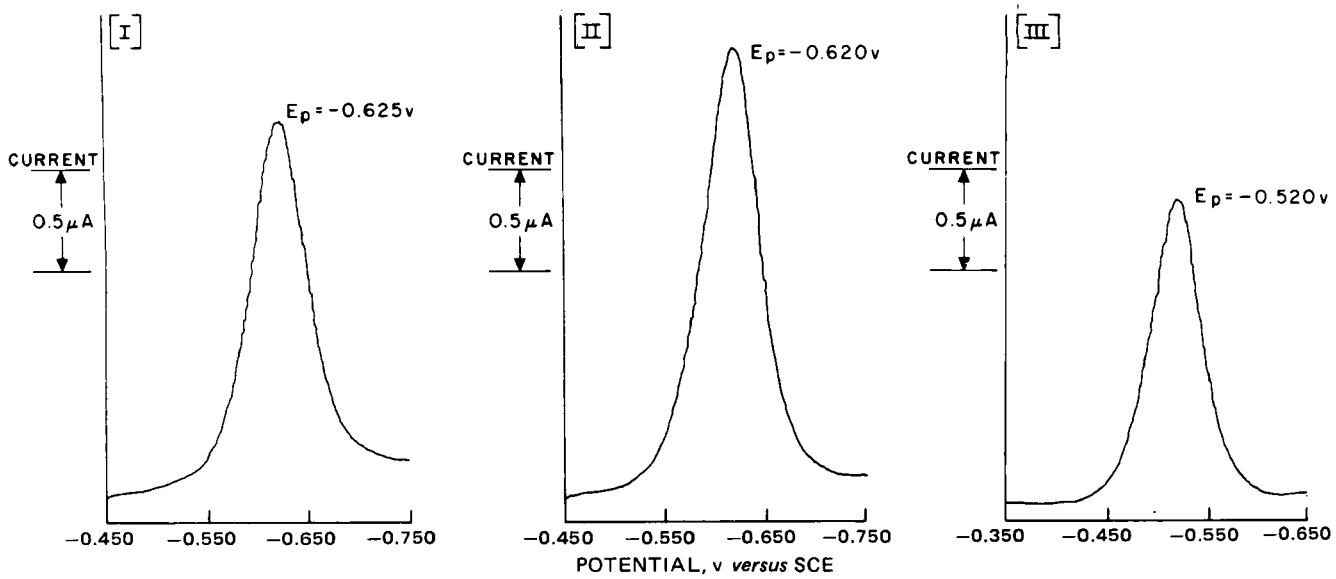

Figure 1—*Differential pulse polarograms of 5 μg of I–III in 3 ml of 0.1* N *NaOH.*

The peak potential, *Ep*, due to the reduction of the nitro group of I, II, and III occurred at −0.625, −0.620, and −0.520 v *versus* the SCE, respectively (Fig. 1).

Reagents—All reagents were analytical reagent grade and were used without further purification. All inorganic reagents were made up in double-distilled water. These included 10 *N* NaOH and 1.0 *M* phosphate buffer (pH 7), made by mixing 390 ml of 1 *M* KH_2PO_4 and 610 ml of 1 *M* K_2HPO_4. Ethyl acetate[4] (spectroquality), chloroform[5], methanol[6], ethanol[7], acetic acid[8], and hydrochloric acid[8] were the other reagents used.

Standard Solutions—Dissolve 10 mg of I, II, or III in 100 ml of absolute ethanol to give a stock solution containing 100 μg/ml. Dilute 1 ml of each stock solution to 10 ml with ethanol to give working solutions containing 10.0 μg/ml. Suitable aliquots of these solutions are added to blood as the internal standards.

Extraction from Plasma—Into a 50-ml glass-stoppered centrifuge tube, add 2 ml of the plasma sample, 6 ml of water, and 2 ml of concentrated hydrochloric acid. Along with the samples, process a 2-ml specimen of control plasma (taken from the same animal prior to medication) and separate 2-ml specimens of control plasma containing 0.5, 1.0, 2.0, 5.0, or 10.0 μg of the compound added as the internal standard. These latter specimens are prepared by evaporating 0.05, 0.1, 0.2, 0.5, and 1.0 ml of the working standards to dryness in a 65° water bath under a stream of nitrogen in 50-ml centrifuge tubes and then adding 2 ml of control plasma, 6 ml of water, and 2 ml of concentrated hydrochloric acid to the residue.

Stir the mixture well on a vortex mixer and place the solution in a water bath at 70° for about 15–20 min. Centrifuge for 10 min in a refrigerated centrifuge at 5° to obtain a clear supernate. If an emulsion is formed, repeat the centrifugation step.

Transfer an 8-ml aliquot into a clean 50-ml tube, and add 2 ml of 1.0 *M* phosphate buffer (pH 7) and sufficient 10 *N* NaOH dropwise to bring the pH of the solution to 7.0 ± 0.2. Extract twice with 10-ml portions of ethyl acetate and combine these extracts in a 15-ml tube by successive evaporation at 65° in a water bath.

TLC Separation—A TLC separation step may be included to ensure assay specificity. Dissolve the residue from the combined ethyl acetate extracts in 100 μl of ethyl acetate and transfer quantitatively onto a 20 × 20-cm silica gel chromatoplate[9]. Develop the plate in a vapor-saturated chamber, using chloroform–acetic acid–methanol (85:10:5), until the solvent front has ascended 15 cm. Examine the plate under shortwave UV light and identify the areas on the silica gel corresponding to I (R_f 0.34), II (R_f 0.55), or III (R_f 0.48) by the R_f values of 10 μg of authentic standards run alongside the sample extracts.

Scrape off these areas and transfer them to a 15-ml centrifuge tube. Add 5 ml of methanol, slurry the tubes on a vortex mixer for approximately 1 min, and centrifuge for 5 min at 2000 rpm to spin down the silica gel. Transfer the supernatant ethanol into another 15-ml conical centrifuge tube and evaporate to dryness in a water bath at 65° under a stream of nitrogen.

Polarographic Analysis—Dissolve the residue from the ethyl acetate extract or from the ethanol supernate in 3 ml of 0.1 *N* NaOH, deoxygenate the sample with nitrogen, and analyze by differential pulse polarography using the described parameters.

Extraction from Urine—Into a 50-ml glass-stoppered centrifuge tube, add 1 ml of urine, 4 ml of water, and 3 ml of 1.0 *M* phosphate buffer (pH 7.0). Adjust the pH to 7.0 ± 0.2 by the dropwise addition of 10 *N* NaOH and extract with ethyl acetate. Prepare internal standards and perform the assay exactly as described for plasma.

Table I—Blood and Urine Levels of Ornidazole (II) in the Dog following 10 mg/kg po[a]

	Plasma Level, μg of II/ml	
Sample Time	Without TLC Separation	With TLC Separation
10 min	11.3	10.5
20 min	10.2	10.3
30 min	9.3	9.7
60 min	10.1	9.2
1.5 hr	10.0	9.1
2.0 hr	8.3	8.5
3.0 hr	8.1	6.7
4.0 hr	7.1	6.2
6.0 hr	5.7	5.1
7.0 hr	3.4	3.5
11.25 hr	1.87	2.0
24 hr	0.55	0.33
30 hr	0.28	0.15
48 hr	0.10	n.m.

	Urine Levels (with TLC Separation)		
	II, μg/ml	Total, mg	Percent of Dose
0–24 hr	12.2	3.09	2.5
24–48 hr	1.35	0.135	0.11

[a] Dose: 122 mg of II in aqueous solution administered orally *via* gastric catheter.

[4] Catalog No. 5G 5212, Matheson, Coleman and Bell, Norwood, Ohio.
[5] Catalog No. C-298, Fisher Scientific Co., Fair Lawn, N.J.
[6] Catalog No. A-412, Fisher Scientific Co., Fair Lawn, N.J.
[7] Pharmaco, Publicker Industries Co., Linfield, Pa.
[8] J. T. Baker Co., Phillipsburg, N.J.
[9] Brinkmann F_{254} silica gel G chromatoplate.

Calculations—The current (microamperes) resulting from each peak and the overall recovery of each compound are determined as previously described (9), while the concentration of each component in the unknowns is determined from the respective internal standard curves.

RESULTS AND DISCUSSION

The polarographic activity of these compounds is attributed to the reduction of their nitro groups. The peaks are pH dependent in that they shift to more negative potential with decreased acidity. The polarographic behavior of the nitroimidazoles was reported previously (10–12).

The overall recovery of I, II, and III from plasma was 60.8 ± 5.4 (*SD*), 59.2 ± 2.6, and 52.7 ± 2.6%. The recovery of the compounds from the silica gel was approximately 90%; thus the overall recovery was approximately 10% lower when the TLC step was included. Extraction of the pure compounds from aqueous solutions under the same conditions as employed in the assay procedure yielded recoveries of 80–85%. Therefore, coprecipitation of the compounds in the formation of the protein-free filtrate from plasma is apparently the major factor resulting in recoveries that are as low as 50%.

The particular protein precipitation reagent and experimental conditions employed in the assay were selected because they yielded an extract for polarographic analysis that was free from interferences originating from the biological matrix. The overall recovery of the three compounds in the urine assay, including the TLC separation step, was approximately 70%. The sensitivity limit of detection was approximately 0.1 μg of I, II, or III/ml of plasma or urine when employing a 2-ml sample.

Specificity—The plasma assay that does not employ a TLC separation step is not specific for the parent drug and determines any extractable metabolite containing a nitro group. However, TLC inspection of the dog plasma extracts for II did not indicate the presence of any metabolite (Table I). This finding may be attributed to the poor extraction of these metabolites, which are probably very polar.

The TLC separation step can be employed effectively to resolve I–III from metabolites, thus establishing the required specificity of the plasma and urine assays. Chronic drug administration may reveal concentrations of metabolites that would require the TLC separation step.

Application to Biological Specimens—Plasma and urine levels were determined in a dog following oral administration of 10 mg/kg of II in aqueous solution *via* gastric catheter. Plasma levels of II employing the assay with and without the TLC separation are reported in Table I. The examination of the plasma extracts under shortwave UV light did not show the presence of any metabolites. The data from the two assays are in good agreement, indicating little interference from metabolites. The plasma levels are also in good agreement with those previously reported (2) for an identical oral dose of II.

The urinary excretion of II was also measured employing the assay with the TLC separation step to separate II from a metabolite noted upon TLC examination. Approximately 2.6% of the administered dose was excreted as unchanged II in the 0–48-hr collection period. This urinary level is lower than that previously reported (2) following an identical oral dose of II.

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