Polarographic Determination of Pyrazinamide Serum and Plasma Concentrations in the Presence of Isoniazid*

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A simple polarographic method for the quantitative determination of pyrazinamide (pyrazine monocarboxyl amide) in human blood serum or plasma is described. The method utilizes a protein-free filtrate obtained by the use of uranyl acetate. Isoniazid (pyridine-3-carboxylic acid hydrazide) does not interfere. Pyrazinoic acid (pyrazine monocarboxylic acid) and pyrazinamide give a single wave, but the individual concentrations may be determined by means of a minimum error potential analysis. The sample can be used directly for isoniazid analysis after pyrazinamide determination.

A RENEWED interest in the use of pyrazinamide in the chemotherapy of tuberculosis has followed reports that the combined therapy of tuberculosis with isoniazid and pyrazinamide is equal or superior to other treatment regimens (1, 2, 3). In following the clinical progress of patients on specific chemotherapy it is desirable to determine, at appropriate intervals, the serum concentration of the chemotherapeutic agents being used. Since serum biological assay procedures are not easily applied in instances where two or more chemotherapeutic agents are being used simultaneously, there is a need for an accurate and rapid chemical method.

A reported colorimetric method requires the use of ion exchange columns (4). The polarographic method described below eliminates such requirements, thereby lending itself more readily to routine clinical analysis. By means of the minimum error potential analysis (5) this polarographic method is also able to distinguish between pyrazinamide and pyrazinoic acid, one of the possible metabolic products, since the amide is readily hydrolyzed in dilute alkali (4).

In the polarographic analysis of a mixture of two reversibly reducible compounds, whose half-wave potentials lie in close proximity to each other, the polarograms do not show sufficiently distinct changes in slope to enable the evaluation of the diffusion current corresponding to each of the individual components. The application of a mathematical interpretation of such a polarogram enables the calculation of the proportionate amounts of each of the components. To accomplish this it is necessary only to determine the potential of minimum error, and measure the current at the potential of minimum error and the current equivalent to the total diffusion current.

EXPERIMENTAL

Materials.—Pyrazine monocarboxyl amide and pyrazine monocarboxylic acid[1] were of a quality designated for investigational use. All other materials used were of reagent quality.

Polarography of Pyrazinamide and Pyrazinoic Acid in Aqueous Solution

To determine the polarographic behavior of pyrazinamide and pyrazinoic acid, solutions of 100 mcg./cc. were prepared and mixed with equal quantities of a universal buffer which contained 0.1 *M* phosphoric, acetic, and boric acids and 0.5 *M* potassium chloride. Various amounts of 1 *M* sodium hydroxide were added to aliquots of the buffer solution to obtain the desired *p*H values. All *p*H measurements were made with a Beckman Model H-2 *p*H meter calibrated at *p*H 4.0 and 7.0 with standard buffers. Polarograms were recorded with a Leeds and Northrup Type E Electrochemograph in conjunction with an H-type cell and a conventional dropping mercury electrode. No attempt was made to obtain precise temperature control since it was noted that room temperature varied only from 24.5° to 25.5°. Oxygen was removed from the test solutions by bubbling for five minutes with nitrogen purified by passage through Fieser's (6) solution. Capillary characteristics at −0.3 v. *vs.* saturated calomel electrode (S.C.E.) showed $m^{2/3}t^{1/6}$ equal to 2.4721.

Appropriate dilutions of a stock solution of pyrazinamide were prepared in the concentration range of 20 to 300 mcg./3 cc. at 20-mcg. increments. Pyrazinoic acid dilutions were prepared in the concentration range of 10 to 100 mcg./cc. at 10-mcg. increments. The cell solution in all cases consisted of 3 cc. of dilution mixed with 3 cc. of aqueous universal buffer. Pyrazinamide solutions were run between −0.30 and −0.60 v. *vs.* S.C.E. Solutions of the acid were run between −0.20 and −0.50 v. *vs.* S.C.E.

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[1] Kindly supplied by Dr. Charles E. Lyght of Merck & Company, Inc.

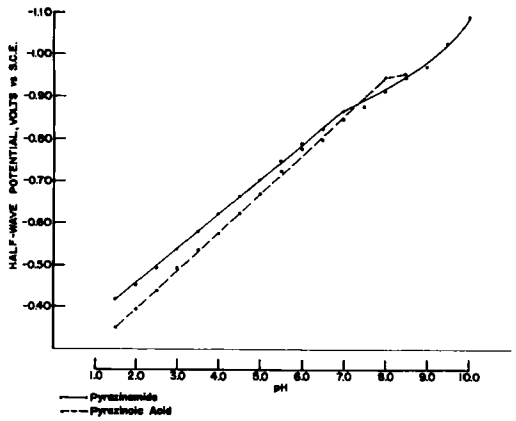

Fig. 1.—Relationship between half-wave potential and *p*H for pyrazinamide and pyrazinoic acid in a universal buffer.

Pyrazinamide was found to exhibit a single, well-defined polarographic wave in the *p*H range of 1.5 to 7.0, after which it no longer exhibited a linear relationship between half-wave potential and *p*H (Fig. 1). The curve for pyrazinoic acid closely parallels that of the amide. The wave of the acid also becomes poorly defined in basic solution.

A *p*H of 1.5 was chosen because a good resolution of the pyrazinamide-pyrazinoic acid wave and the two succeeding waves of isoniazid (7) was obtained (Fig. 2). At this *p*H, pyrazinamide alone exhibited a half-wave potential of −0.417 v. *vs.* S.C.E., whereas that of the acid alone was −0.362 v. *vs.* S.C.E. in aqueous solution.

The linear relationship between diffusion current and concentration for both pyrazinamide and pyrazinoic acid is shown in Fig. 3. Linearity is a prerequisite for the application of the minimum error potential analysis. The height of the mercury column above the tip of the capillary must remain constant for the relationship to hold.

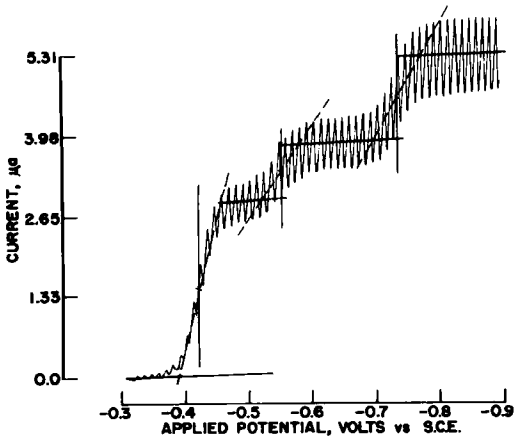

Fig. 2.—A typical polarogram of pyrazinamide in the presence of isoniazid in aqueous solution of *p*H = 1.5 (37.5 mcg. pyrazinamide/cc.; 14.3 mcg. isoniazid/cc.).

Minimum Error Potential Analysis of Aqueous Solutions.—Providing the condition of noninteraction of the two components of a binary mixture is met, the fundamental equation of Heyrovský and Ilkovič is valid for such a mixture. This equation can then be presented in the form of Eq. 1:

$$(A) = \frac{(id_{AB}K_B - i_{AB})}{(K_B - K_A)(K'_A)} \text{ and } (B) = \frac{(i_{AB} - id_{AB}K_A)}{(K_B - K_A)(K'_B)} \quad (1)$$

Where A and B are the concentrations of the two components, id_{AB} is the total diffusion current assuming additivity, i_{AB} is the current reading at the predetermined potential of minimum error, K'_B and K'_A are the constants derived for each of the pure compounds if the linear relationship between diffusion current and concentration holds for each

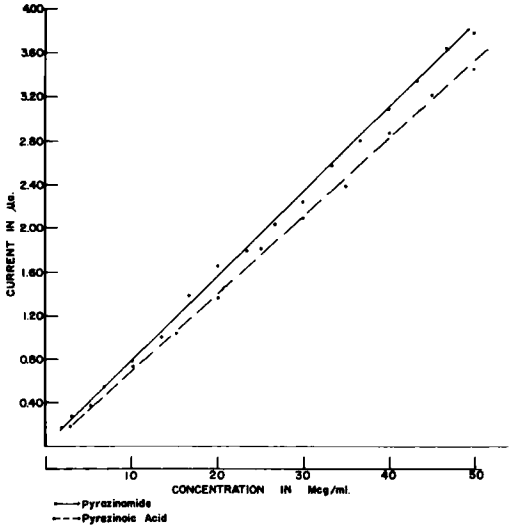

Fig. 3.—Diagram showing the linear relationship between current and concentration of pyrazinamide and pyrazinoic acid in aqueous solution at *p*H of 1.5. (All points are the average values of duplicate determinations.)

component, and K_B and K_A are the constants at any one potential, in this case, the potential of minimum error. A complete derivation may be found in the work of Frisque, *et al.* (5).

When the same electron change is undergone by both components ($n_A = n_B$), it follows that the minimum error potential is defined by Eq. 2:

$$E = {}^1/_2(E_{1/2B} + E_{1/2A}) \quad (2)$$

It was found that the reduction of both the acid and the amide was a two electron reaction, giving a minimum error potential in aqueous solution of −0.390 v. *vs.* S.C.E.

Additivity of the individual diffusion currents of the acid and amide when mixed was found within the limits of ±0.08 µa over the entire concentration range studied.

Tables I and II give the experimental values employed on synthetic mixtures. Table III gives the

single potential constants for each component present in mixtures and the difference between the constants as a function of applied potential.

TABLE I.—EXPERIMENTAL VALUES FOR PYRAZINOIC ACID EMPLOYED IN CALCULATIONS ON SYNTHETIC MIXTURES

Concentration, mcg./ml.	$E_{1/2}$	i_dPyr. Acid (μa)	K' Pyr. Acid
50	−0.364	3.46	0.0692
40	−0.363	2.87	0.0714
30	−0.362	2.10	0.0700
20	−0.361	1.37	0.0685
10	−0.360	0.73	0.0730
Av.	−0.362		0.0704 = K'_B

TABLE II.—EXPERIMENTAL VALUES FOR PYRAZINAMIDE EMPLOYED IN CALCULATIONS ON SYNTHETIC MIXTURES

Concentration, mcg./ml.	$E_{1/2}$	i_dPyr. (μa)	K'Pyr.
50	−0.417	3.78	0.0756
40	−0.418	3.09	0.0773
30	−0.415	2.24	0.0747
20	−0.416	1.66	0.0830
10	−0.417	0.78	0.0780
Av.	−0.417		0.0777 = K'_A

TABLE III.—CONSTANT[a] FOR EACH COMPONENT PRESENT IN MIXTURES AND DIFFERENCE BETWEEN CONSTANTS AS A FUNCTION OF APPLIED POTENTIAL

Applied Potential, v.	K_B	K_A	$(K_B - K_A)$
−0.330	0.077	0.001	0.076
−0.360	0.465	0.014	0.451
−0.380	0.806	0.052	0.754
−0.390	0.901	0.107	0.794
−0.400	0.952	0.208	0.744
−0.430	0.990	0.901	0.089
−0.460	1.000	0.971	0.029

[a] Single potential constant

$$K = 1/[1 + 10\,(E - E_{1/2})\,n/0.059]$$

K_A = K (Pyrazinamide)
K_B = K (Pyrazinoic acid)
n = 2 for both compounds
$E_{1/2}$(Pr) = −0.362
$E_{1/2}$(Pyracid) = −0.417

The necessity of determining the potential of minimum error is obvious. Any small error in the determination of either K_A or K_B will cause a relatively smaller error in the concentrations (A) and (B) if the difference between the two constants is large. Thus, the current i_{AB} should be measured at the potential at which the quantity $(K_B - K_A)$ is at a maximum.

Since $(K_B - K_A)$ is a maximum when i_{AB} is evaluated at an applied potential of −0.039 v. *vs.* S.C.E., the differences between the theoretical and experimental values for the amount of pyrazinamide and pyrazinoic acid present in each mixture should be at a minimum value when i_{AB} is measured at this potential. Synthetic mixtures of pyrazinamide and pyrazinoic acid were prepared at a total concentration of 100 mcg./ml. with ratios of 10:90 to 90:10 at 10-mcg. increments for each component. Three cubic centimeters of each solution was mixed with 3 cc. of universal buffer at *p*H 1.5. (Final concentration ratios at 5:45 to 45:5 at 5-mcg. increments.) All solutions were treated as above and polarographed between −0.20 and −0.55 v. *vs.* S.C.E. Table IV gives a comparison of the theoretical and experimental results obtained on such synthetic mixtures.

Such a method for the determination of binary mixtures employing the described procedure has obvious limitations. The resulting error in experimentally determined values for the amount of each component present becomes larger as the ratio of the half-wave potentials of the two components approaches unity. It must be further emphasized that since the minimum error potential method assumes a single clearly defined half-wave potential for each component in the applied potential range of interest, the analysis, therefore, will not hold when a stepwise exchange of electrons occurs for one of the components at nearly similar voltages.

Selection of a Protein Precipitant.—Polarographic determinations made directly on serum or plasma showed the wave of pyrazinamide to be absent. Associated with this behavior was the observation that the individual mercury droplets did not coalesce in the bottom of the electrolysis cell. Recurrent observation of these phenomena was attributed to incomplete protein removal.

Since the preparation of an extract was considered to be too time-consuming for routine analysis, a number of protein-precipitating reagents were investigated to find one which would not give an interfering wave in the potential range of −0.2 v. to

TABLE IV.—THEORETICAL AND EXPERIMENTAL VALUES FOR PYRAZINAMIDE AND PYRAZINOIC ACID IN SYNTHETIC MIXTURES OF THE TWO COMPOUNDS

Pyrazinamide			Pyrazinoic Acid		
Theor., mcg./ml.	Exptl., mcg./ml.	Error, %	Theor., mcg./ml.	Exptl., mcg./ml.	Error, %
0.0	3.9	...	50.0	49.0	− 2.0
5.0	5.9	+18.0	45.0	42.3	− 6.0
10.0	11.0	+10.0	40.0	40.6	+ 1.5
15.0	11.3	−24.7	35.0	38.6	+10.3
20.0	21.2	+ 6.0	30.0	30.0	0.0
25.0	25.2	+ 0.8	25.0	25.9	+ 3.6
30.0	32.2	+ 7.3	20.0	20.2	+ 1.0
35.0	36.7	+ 4.9	15.0	9.9	−34.0
40.0	40.0	0.0	10.0	9.3	− 7.0
45.0	40.8	− 9.3	5.0	6.8	+36.0
50.0	48.0	− 4.0	0.0	0.7	...
	Av. rel. error	8.5		Av. rel. error	10.1

TABLE V.—POLAROGRAPHIC CHARACTERISTICS OF PROTEIN PRECIPITANTS INVESTIGATED

Compound	Characteristics
Trichloroacetic acid (20%)	An interfering catalytic-type wave between −0.3 v. and −0.8 v. *vs.* S.C.E.
Tungstic acid (10%) (sodium salt with sulfuric acid)	Wave of $E_{1/2}$ at −0.69 v. *vs.* S.C.E., with increasing residual current noted starting at −0.3 v. *vs.* S.C.E., which covered the pyrazinamide wave at low concentrations.
Absolute ethanol	Incomplete protein precipitation. (Hg droplets do not coalesce.)
Metaphosphoric acid (20%)	Incomplete protein precipitation. (Hg droplets do not coalesce.)
Tannic acid (20%)	Excellent precipitant; mercury droplets coalesce; pyrazinamide wave has $E_{1/2}$ shifted to −0.52 v. *vs.* S.C.E.; low residual current and good wave obtained.
Uranyl ion (saturated solution as the acetate)	Excellent precipitant; Hg droplets coalesce; low residual current and good wave obtained.

−0.6 v. *vs.* S.C.E. The commonly used protein precipitants were found to interfere in this range. Table V lists the protein precipitants investigated and the polarographic characteristics observed.

The results presented in Table V indicate tannic acid to be as efficient a protein precipitant as uranyl acetate. However, the excess tannic acid prevented the subsequent use of vanillin for isoniazid determination (8, 9). Uranyl acetate was found to be a suitable substitute for trichloroacetic acid, necessitating only the preparation of new standard curves. The use of this procedure had the added advantage of using a minimum of serum for determination of more than one chemotherapeutic agent.

Polarography of Pyrazinamide in Protein-Free Plasma or Serum Centrifugates

A stock solution of 100 mg. pyrazinamide/100 cc. plasma or serum was prepared. Dilutions of 10 to 150 mcg./cc. at 10-mcg. increments were prepared by further dilution with pooled plasma or serum. Standard curves were prepared for an original serum or plasma volume of 1 cc. and 3 cc. The samples were then treated in the following manner.

(*a*) One cubic centimeter of serum or plasma (3 cc.) was mixed with 1 cc. (3 cc.) of saturated uranyl acetate in a 15-cc. centrifuge tube and centrifuged for twenty minutes at 1800 G (10).

TABLE VI.—COMPARISON OF THEORETICAL RECOVERIES OF SERUM AND PLASMA PYRAZINAMIDE TO ACTUAL RECOVERIES[a]

Orig. Concn., mcg./cc.	1-cc. Sample			3-cc. Sample		
	Theor., mcg./cc.	Found, mcg./cc.	Recovery, %	Theor., mcg./cc.	Found, mcg./cc.	Recovery, %
10	1.7	0.3	17.7	4.8	2.0	41.7
20	3.5	1.0	28.6	9.7	4.3	44.3
30	5.2	1.7	32.6	14.5	6.7	46.2
40	6.9	2.3	33.4	19.4	9.7	50.0
50	8.7	3.3	37.9	24.2	11.3	46.7
60	10.4	4.0	38.4	29.0	14.7	50.7
70	12.1	5.0	41.7	33.9	16.7	49.3
80	13.8	6.0	43.5	38.8	18.3	47.2
90	15.6	7.0	44.9	43.6	21.0	48.1
100	17.3	8.0	46.2	48.4	22.7	47.0
110	19.0	8.7	45.8	53.2	26.7	50.2
120	20.8	9.3	44.7	58.1	29.3	50.4
130	22.5	9.7	43.1	62.9	31.7	50.4
140	24.2	10.7	44.2	67.8	34.0	50.2
150	26.0	11.3	43.5	72.6	35.3	48.6
		Av. recovery =	38.4			48.0

[a] Theoretical calculated as original concentration/final volume for 1-cc. and 3-cc. samples. Average final volumes are 5.8 cc. and 6.2 cc. respectively.

TABLE VII.—COMPARISON OF THEORETICAL AND ACTUAL RECOVERIES OF PYRAZINAMIDE FROM A 3-CC. SAMPLE[a]

Orig. Concn., mcg./cc.	Theor. (Orig./6.2 cc.), mcg./cc.	Found, mcg./cc.	Recovered, %	Theor. 1st Wash (Theor.—Found/5.9 cc.), mcg./cc.	Found, mcg./cc.	Recovered, %	Total Recovered, %
10	4.8	2.1	43.8	2.9	1.0	34.4	63.0
30	14.5	6.4	44.1	8.5	3.7	43.5	68.3
50	24.2	10.6	43.8	14.3	4.3	30.1	60.7
100	48.4	21.5	44.4	28.3	8.9	31.4	61.9
150	72.6	34.9	48.1	39.6	13.9	35.1	66.3

[a] Theoretical calculated as in Table VI; theoretical of 1st wash calculated as difference between theoretical and found for original protein precipitation.

TABLE VIII.—ANALYSIS OF PATIENT SERUM POLAROGRAMS[a]

Patient No.	Concn. in mcg./ml. at Designated Hour After Oral Administration																	
	1 hr.			2 hr.			3 hr.			7 hr.			8 hr.					
	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP)	PZA (Tot. Wave Ht.)	Pyr. Acid (MEP)	PZA (MEP) (a)	PZA (MEP) (b)	PZA (Tot. Wave Ht.) (a)	PZA (Tot. Wave Ht.) (b)	Pyr. Acid (MEP) (a)	Pyr. Acid (MEP) (b)
1	13.4	13	0.0	...[b]	..	...	...	..	...	12.9	13	0.6	15.9	..	15	..	0.0	...
2	53.9	54	1.6	47.4	48	1.6	33.2	40	5.6	28.9	32	2.8	35.3	31.0	35	34	0.6	3.4
3	25.4	25	0.6	33.2	34	1.2	19.8	21	1.6	5.6	6	0.0	17.7	0.0	17	0	0.6	0.0
4	24.6	24	0.0	...	..	...	...	..	...	...	..	...	4.3	...	4	..	0.0	...
5	44.4	46	1.6	98.8	92	1.2	66.4	70	4.0	58.6	54	0.0	51.7	78.4	51	77	0.0	0.0
6	32.8	32	0.0	...	..	...	24.6	30	4.4	19.0	21	1.2	19.0	20.7	19	22	0.6	1.2
7	33.2	34	1.6	34.5	37	2.2	23.3	29	4.4	19.0	21	1.2	24.1	26.3	25	28	1.6	1.6
8	42.7	42	0.6	...	..	...	...	..	...	24.6	25	1.2	21.1	22.8	21	23	0.0	0.6
9	...	..	...	46.5	49	3.4	...	..	...	20.7	21	1.2	24.6	...	25	..	0.6	...
10	46.5	48	1.6	51.7	53	1.6	38.8	41	2.2	22.8	24	1.6	32.7	34.0	33	35	0.6	1.6
11	38.8	41	2.8	36.2	40	3.4	24.6	30	4.4	19.8	22	2.2	23.3	25.4	26	30	1.6	4.0
12	49.6	50	1.2	...	..	...	29.7	34	4.0	26.3	26	0.6	28.9	25.4	29	25	0.0	0.6
13	31.9	33	0.6	35.3	38	3.4	30.6	33	2.8	26.3	26	1.2	27.6	26.7	27	28	0.0	1.2
14	28.9	29	0.0	24.1	26	2.2	24.6	25	1.2	16.8	17	0.6	19.0	12.9	18	13	0.0	0.6
15	64.2	65	1.6	54.3	58	3.4	40.5	48	7.5	36.2	39	2.8	46.1	...	46	...	1.2	...

[a] Pyrazinoic acid calculated by the minimum error potential method. Pyrazinamide calculated both by the minimum error potential method and by assuming no hydrolysis and thus total wave height due to pyrazinamide.
[b] Missing figures due to inability to obtain serum sample.

Fig. 4.—Standard curve for pyrazinamide concentration of protein-free plasma or serum centrifugation. (All points are the average values of duplicate determinations.)

(*b*) The yellow-colored centrifugate was decanted into another 15-cc. centrifuge tube and mixed with 3 cc. (both original sample volumes) of universal buffer of *p*H 1.5.

(*c*) The resulting flocculent suspension of slightly soluble alkali uranyl phosphate was centrifuged again for twenty minutes at 1800 G (10), then 2 cc. of distilled water was added to bring the total volume of clear centrifugate to more than 5 cc. Where the original sample was 3 cc., no addition of water was necessary.

(*d*) Five cubic centimeters of the clear supernatant was then pipetted into the electrolysis vessel and after deoxygenating for five minutes, the pyrazinamide concentration was determined and the sample recovered.

Although the standard curves represented in Fig. 4 were prepared from plasma, it was found that identical values were obtained with serum.

Fig. 5.—Typical polarogram of patient serum concentration of pyrazinamide, demonstrating the inability to measure isoniazid concentration polarographically (28 mcg. pyrazinamide/cc. serum; 5.2 mcg. isoniazid/cc. serum).

The advantage in using as large a plasma or serum sample volume as possible was to minimize the dilution factor. It was found necessary to obtain a filtrate volume of greater than 5 cc. for the H-type electrolysis vessel used. This volume necessitates an approximate dilution factor of 1:6 for a 1-cc. sample and 1:2 for a 3-cc. sample. Since the recoveries are of the order of 40 to 50% for a 3-cc. sample as compared to 17 to 45% for a 1-cc. sample, as shown in Table VI, this is a further indication of the desirability of using a larger serum or plasma sample.

Although the recoveries were found to be low, the consistency (±6% in the patient serum concentration range of 20 to 50 mcg./cc.) was considered adequate to give accurate results. However, further information regarding the remainder of the pyrazinamide was deemed advisable. Accordingly, another series of samples was prepared and treated as above. The original protein precipitate was then washed with 3 cc. of distilled water and recentrifuged. Three cubic centimeters of the centrifugate was then mixed with 3 cc. of universal buffer and treated as above. This was considered as the first wash. The procedure was repeated to obtain the second wash. Table VII gives the results of the additional recovery.

The second wash was found to give a poorly defined wave, which could not be accurately measured. Noncoalescence of the mercury droplets was noticed in the majority of these cases. This was attributed to protein solubility upon repeated washing. No advantage could be seen in incorporating the washing technique into the procedure, since it doubled the volume and did not result in a corresponding increase in recovery of pyrazinamide.

It might seem that a concurrent polarographic determination could also be made for isoniazid. However, comparison of the aqueous (Fig. 2) and protein-free serum-centrifugate polarograms (Fig. 4) show a marked difference in isoniazid concentration. As long as patient serum isoniazid concentrations are of the order of 0.1 to 15 mcg./ml. (9), a further dilution in obtaining a protein-free filtrate gives a final isoniazid concentration too low to measure accurately polarographically in the cited system.

Minimum Error Potential Analysis of Protein-Free Filtrates.—It must be assumed that hydrolysis of the amide as a means of detoxification *in vivo* could occur. This necessitated the determination of the potential of minimum error in a protein-free serum filtrate system. Pyrazinamide was found to exhibit a half-wave potential of −0.439 v. *vs.* S.C.E. while that of the acid was −0.383 v. *vs* S.C.E. This resulted in a minimum error potential of −0.406 v. *vs.* S.C.E. A recalculation of the single potential constants K_A and K_B for a protein-free filtrate system gave values of 0.071 and 0.855, respectively. Table VIII gives the results of the analyses of patient serum polarograms.

The determination of the absolute concentration of both the amide and the acid necessitates consideration of both the dilution and recovery factors. For pyrazinamide the average recovery is 48%. The recovery of pyrazinoic acid in the concentration range of 5 to 10 mcg./cc. is 66.7%. The correction for the dilution factor for both is 6.2 cc./3 cc. Thus, the factors for the acid and the amide are 3.11 and 4.31, respectively. These are the quantities which must be multiplied by the concentration determined by the minimum error potential analysis, in order to obtain the absolute value for the concentration/cc. of patient serum.

It can be observed in Table VIII that little or none of the amide undergoes hydrolysis *in vivo* in the 1 to 8-hour period of study. For this reason, it can be assumed for clinical evaluation, that the entire wave height is due to pyrazinamide and an analysis for the acid content is not necessary. This then allows the simple comparison of experimental *ia* values with a standard curve, from which pyrazinamide concentrations may be read directly.

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