Automated Analysis of Indapamide in Drug–Rodent Food Mixtures

MARTHA M. JOHNSTON ˣ, MARC J. ROSENBERG, ANNE K. YEUNG *, and PETER E. GREBOW

Received July 23, 1979, from the *Biochemistry and Drug Disposition Department, USV Pharmaceutical Corporation, Tuckahoe, NY 10707.* Accepted for publication April 10, 1980. *Present address: Lederle Laboratories, Pearl River, NY 10965.

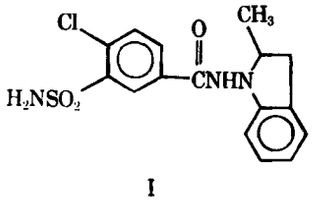
Abstract □ Indapamide, an antihypertensive agent, is an aryl sulfonamide that inhibits carbonic anhydrase *in vitro* but not *in vivo*. An assay was developed for indapamide in drug–rodent food mixtures that utilizes this inhibitory effect. Indapamide was extracted from the mixtures with methanol, and an aqueous dilution of the extract was sampled by a continuous-flow system. In the system, the drug was extracted with butanol and then back-extracted into alkali. This solution was neutralized, buffered, and mixed with bovine erythrocyte carbonic anhydrase. The substrate, *p*-nitrophenyl acetate, was added, the solution was incubated, and the amount of *p*-nitrophenol formed was measured. The assay was sensitive to 20 μg of indapamide/g of food, and 20 unknown samples could be analyzed per hour on the continuous-flow system. It is possible that the method could be extended to the analysis of other toxicological test substances that inhibit carbonic anhydrase *in vitro*.

Keyphrases □ Indapamide—automated analysis in drug–rodent food mixtures, carbonic anhydrase inhibitor □ Antihypertensive agents—indapamide, automated analysis in drug–rodent food mixtures □ Carbonic anhydrase inhibitors—indapamide, automated analysis in drug–rodent food mixtures

Indapamide[1] [4-chloro-*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide, I] is an antihypertensive agent (1). It is an aryl sulfonamide, and compounds in this class inhibit carbonic anhydrase (2). The inhibition of carbonic anhydrase by indapamide (3) occurs under *in vitro* conditions only, and there is no evidence of *in vivo* inhibition after therapeutic doses (1).

Since an automated assay based on the inhibition of carbonic anhydrase was developed for chlorthalidone (4), the procedure was considered as a possible method for the analysis of the indapamide content in drug–rodent food mixtures in toxicological studies. A fluorometric assay for indapamide was developed (5), but it was time consuming when used with a large number of samples. Thus, the automated method was modified because it had the potential for the greatest rate of sample processing.

The principle of the enzymatic assay is based on the catalytic hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol by carbonic anhydrase. The rate of formation of *p*-nitrophenol is a measure of the uninhibited enzymatic velocity. The presence of indapamide inhibits the enzyme, causing a decrease in the enzymatic velocity and in the rate of *p*-nitrophenol formation. In the assay, the decrease in the *p*-nitrophenol formation rate, measured at 400 nm, is proportional to the concentration of indapamide present.



I

[1] USV 2555.

EXPERIMENTAL

Reagents—The buffer was 0.1 *M* tris(hydroxymethyl)aminomethane[2] (pH 8.0). The enzyme solution contained 40 mg of bovine erythrocyte carbonic anhydrase[2]/liter of buffer plus 0.1% surfactant[3]. The substrate solution was 2.0 m*M* *p*-nitrophenyl acetate[2] in polyethylene glycol 200[4]. The butanol was saturated by shaking five volumes of butanol[5] with two volumes of glass-distilled, deionized water. Solutions of 0.01 *N* NaOH[6] and 0.1 *N* HCl[6] were used.

Indapamide Standards—Control rodent food[7] was extracted with methanol[8] (1 g of food/10 ml of methanol), and the methanol supernate was diluted 1:10 with water. This 10% methanol solution was used to prepare indapamide[9] standards of 0.0, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, and 8.0 μg/ml.

Extraction—Samples (~0.50 g) of the indapamide–rodent food mixture were weighed, shaken for 30 min with 5.0 ml of methanol, and centrifuged for 10 min. A 1.0-ml aliquot of the methanol supernate then was diluted to 10.0 ml with glass-distilled, deionized water for the final solution, which was to be sampled automatically.

Automated Procedure—Figure 1 is a diagram of the continuous-flow system[10]. The baseline was adjusted to zero as the buffer plus 0.1% surfactant was pumped through the enzyme line and the other reagents were pumped through their respective lines. After a stable baseline was present for 5 min, the enzyme line was switched to the enzyme solution. After the large increase in absorbance was recorded, indicating that the enzyme had traversed the entire system, the standard calibration of the spectrophotometer was adjusted so that the enzymatic baseline was between 50 and 60 relative absorbance units on the chart.

After the enzymatic baseline had been established for 5 min, sampling was initiated; inhibition of the carbonic anhydrase by the samples was determined by measuring the decrease in absorbance (ΔA) (Fig. 2). Standards were run before and after every 15–20 unknown samples, and the indapamide concentration of the extract was calculated from a standard curve of ΔA *versus* the indapamide concentration. The indapamide content of the food mixture was calculated from:

$$\text{mg of indapamide/g of food} = 0.05 \times (\mu\text{g of indapamide/ml of extract})/\text{g of sample} \quad \text{(Eq. 1)}$$

where 0.05 is a dilution factor with units of (milliliters × milligrams) per microgram.

RESULTS AND DISCUSSION

Standard Curve—Figure 2 shows a typical recording obtained with a set of standards, and Table I contains the results of triplicate analyses of the standards. A plot of the data in Table I yielded a slightly curved standard curve, a phenomenon that was observed previously (6) and that may be due to the complex interaction of the kinetic constants defining the interaction of the enzyme with the substrate, *p*-nitrophenyl acetate,

[2] Sigma Chemical Co., St. Louis, Mo.
[3] Triton X-405, Rohm & Haas Co., Philadelphia, Pa.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Fisher Scientific Co., Pittsburgh, Pa.
[6] Mallinckrodt, St. Louis, Mo.
[7] Ralston-Purina, St. Louis, Mo.
[8] Burdick & Jackson Laboratories, Muskegon, Mich.
[9] Servier Laboratories, Neuilly Sur Seine, France.
[10] AutoAnalyzer II, sampler IV, proportioning pump III, UV spectrophotometer, and recorder, Technicon Industrial Systems, Tarrytown, N.Y.

0022-3549/80/1000-1158$01.00/0

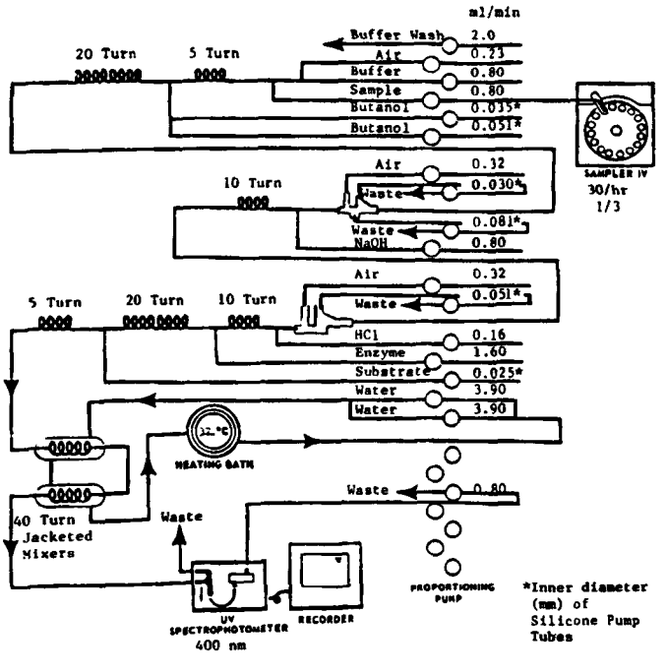


Figure 1—*Continuous-flow system.*

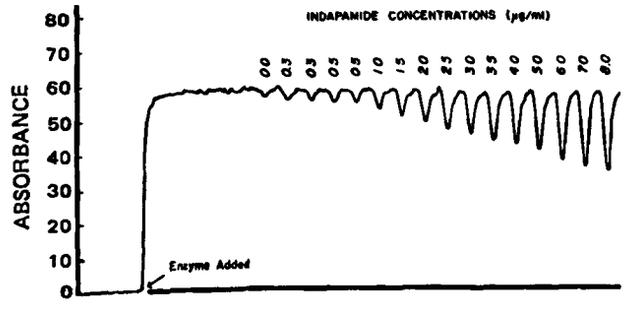


Figure 2—*Typical chart recording of a series of standard samples. Inhibition of the enzyme by the samples caused the decrease in absorbance, ΔA.*

and the inhibitor, indapamide. The ΔA values of unknown samples can be converted to concentrations by reading the values from the standard curve manually, or, since the curvature of the standard curve is slight, the standard values that bracket the unknown values can be used to construct a straight line whose parameters (slope and intercept) can be used to convert the unknown ΔA values to concentrations by a computer program.

For example, a regression analysis of the data in Table I from 0 to 3.0 µg/ml yielded a correlation coefficient (r) of 0.998 with a standard error of 0.234, corresponding to an error of ±0.067 µg of indapamide/ml. This line segment was used for unknown samples with concentrations of <3.0 µg/ml. For higher concentrations, the upper portion of the standard curve was used, which had a correlation coefficient of 0.996 and a standard error of 0.337, corresponding to an error in the indapamide concentration of ±0.147 µg/ml.

Table I—Analysis of Standards

Indapamide Concentration, µg/ml	ΔA, Mean ± SD[a]
0.0	2.5 ± 0.0
0.3	3.5 ± 0.1
0.5	4.4 ± 0.2
1.0	6.0 ± 0.2
1.5	8.1 ± 0.1
2.0	9.7 ± 0.1
2.5	11.2 ± 0.1
3.0	12.9 ± 0.5
3.5	14.6 ± 0.1
4.0	15.7 ± 0.1
5.0	17.7 ± 0.2
6.0	19.8 ± 0.6
7.0	22.0 ± 0.0
8.0	24.9 ± 0.4

[a] Average of standards assayed three times.

Table II—Precision of Analysis of Unknown Indapamide–Food Mixtures

Batch Number	Sample Weight, g	Indapamide Concentration, µg/ml	Indapamide Content, mg/g	Mean Indapamide Content ± SD, mg/g
121	0.504	0.486	0.048	0.048 ± 0.001
	0.505	0.486	0.048	
	0.502	0.478	0.047	
110	0.505	0.803	0.080	0.083 ± 0.005
	0.505	0.812	0.080	
	0.502	0.905	0.090	
249	0.504	1.737	0.172	0.187 ± 0.042
	0.504	2.379	0.236	
	0.501	1.531	0.153	
325	0.503	4.328	0.430	0.427 ± 0.007
	0.503	4.359	0.433	
	0.506	4.244	0.419	
235	0.507	5.672	0.559	0.541 ± 0.031
	0.506	5.652	0.558	
	0.505	5.105	0.505	
301	0.506	5.944	0.587	0.580 ± 0.028
	0.503	6.082	0.605	
	0.503	5.525	0.549	

Table III—Precision of Analysis of Analytically Prepared Indapamide–Food Mixtures

Indapamide Content as Prepared, mg/g	ΔA[a]	Indapamide Content as Analyzed[a], mg/g
0.5	4.97 ± 0.10 (4.8–5.1)	0.504 ± 0.022 (0.466–0.540)
5.0	17.73 ± 0.53 (17.0–19.0)	4.92 ± 0.21 (4.63–5.42)

[a] The values are expressed as the mean and standard deviation of 12 determinations. The range of values is in parentheses.

Precision of Assay—Triplicate samples were taken from several batches of indapamide–rodent food mixtures and were weighed and extracted. Each extracted sample was sampled and assayed by the continuous-flow system three times. The results are shown in Table II. The overall precision of the assay was calculated to be ~7%.

To ensure the precision of the assay by precluding possible variations due to nonhomogeneous mixing of the drug–food samples, 25 or 250 µl of a 1.0-mg/ml indapamide solution was added to 0.5 g of the powdered food. This addition was done to 12 tubes at each concentration to give samples corresponding to 0.5 and 5.0 mg of drug/g of food. The samples were extracted and assayed as described. Table III shows the mean standard deviation and range of the ΔA values and their corresponding indapamide content as determined analytically for the two prepared concentrations.

Table IV—Analysis of Samples by Two Independent Methods

Batch	Indapamide Content, mg/g		Difference, mg/g
	Enzymatic[a]	Fluorescence[b]	
103	0.048	0.049	−0.001
105	0.044	0.061	−0.017
111	0.053	0.049	0.004
121	0.047	0.048	−0.001
311	0.515	0.437	0.078
321	0.521	0.472	0.049
329	0.506	0.486	0.020
335	0.456	0.495	−0.039

[a] Assayed using the inhibition of carbonic anhydrase. [b] Assayed by the fluorometric method of Ref. 5.

Sensitivity—Fourteen batches of rodent food that had not been mixed with indapamide were extracted and analyzed. The apparent indapamide content ranged from −0.010 to +0.017 mg/g, and the mean and standard deviation of the 14 values were 0.000 ± 0.008 mg/g. This result indicates that an indapamide–food mixture containing ≥0.02 mg/g can be analyzed by this method.

Selectivity—There was little variation in the content of endogenous inhibitory compounds, as shown by the data on the 14 batches of drug-free food. The magnitude of the inhibition was small (Fig. 2). Table IV shows the results of the analysis of indapamide-containing samples by the enzymatic method described in this report and by the fluorescence method described previously (5). The results indicate agreement between the two methods.

Exogenous compounds that contain an aryl sulfonamide group are likely to inhibit carbonic anhydrase (2, 3, 6) and to interfere with the assay. However, in a well-controlled toxicological study, they would not be present. Nevertheless, this lack of specificity could be very useful because this method possibly could be used for other substances containing an aryl sulfonamide group. Thus, a method has been developed for the analysis of indapamide in drug–rodent food mixtures by using a simple extraction followed by an automated system capable of analyzing ~20 unknown samples/hr. In addition, the method can be used for other test substances.

REFERENCES

(1) M. Laubie and H. Schmitt, *Curr. Med. Res. Opin., Suppl. 1*, **5**, 89 (1977).

(2) T. Mann and D. Keilen, *Nature*, **146**, 164 (1940).

(3) M. Khiat, J. Bali, M. Guibest, J. Chanal, and R. Marignan, *Clin. Chim. Acta*, **82**, 241 (1978).

(4) M. Johnston, M. Rosenberg, and B. Kamath, *J. Pharm. Sci.*, **68**, 967 (1979).

(5) P. Grebow, J. Treitman, and A. Yeung, *ibid.*, **67**, 1117 (1978).

(6) M. Johnston, H. Li, and D. Mufson, *ibid.*, **66**, 1735 (1977).

ACKNOWLEDGMENTS

Presented in part at the APhA Academy of Pharmaceutical Sciences, Hollywood, Fla. meeting, November 1978.

Effects of *cis*-Malonato-diammino Platinum(II) on P-388 Lymphocytic Leukemia Cell Metabolism

I. H. HALL ˣ, M. H. HOLSHOUSER, and L. J. LOEFFLER

Received January 23, 1980, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514.* Accepted for publication April 2, 1980.

Abstract □ *cis*-Malonato-diammino platinum(II) significantly inhibited P-388 lymphocytic leukemia cell proliferation at 10 mg/kg/day. Incorporation studies showed that DNA synthesis was inhibited following *in vivo* drug therapy. The major inhibitory effects appeared to be on thymidine kinase and dihydrofolate reductase activities and on overall purine synthesis, with marginal effects on DNA polymerase and ribonucleotide reductase activities. In addition to the DNA inhibition, a marked increase in cyclic adenosine 3′,5′-monophosphate levels was noted, which correlated with a rapid decrease in histone phosphorylation. Other minor effects of the drug included significant reduction of proteolytic activity, suppression of States 4 and 3 respiration, and an increase in adenosine triphosphatase and acid phosphatase activities of P-388 cells.

Keyphrases □ *cis*-Malonato-diammino platinum(II)—synthesis and evaluation for antineoplastic activity *in vivo* and *in vitro*, mice □ Antineoplastic agents—*cis*-malonato-diammino platinum(II), synthesis and evaluation for antineoplastic activity *in vivo* and *in vitro*, mice □ Platinum—synthesis of *cis*-malonato-diammino platinum(II) and evaluation for antineoplastic activity *in vivo* and *in vitro*, mice

Rosenberg *et al.* (1) observed that certain coordination compounds of platinum possessed antibacterial activity. The antineoplastic activity of *cis*-1,2-dichlorodiammine platinum(II) initially was delineated against sarcoma 180 (2) and plasma cell myeloma (PC 6) (3) cell proliferation in mice. Problems associated with *cis*-1,2-dichlorodiammine platinum(II) are its low aqueous solubility and toxicity, *e.g.*, nephrotoxicity, nausea and vomiting, occasional neuromuscular toxicity, ophthalmological toxicity and ototoxicity, anaphylaxis, and allergic reactions. These effects limit the clinical use of the drug (4, 5) and led to chemical modification of this agent, resulting in more than 1000 new individual complexes. However, only 80 agents have demonstrated significant antineoplastic activity (5).

Cleare and Hoeschele (6) observed that substitution of the chloride ions with a bidentate ligand, *e.g.*, *cis*-malonato-diammino platinum(II), resulted in potent antineoplastic activity against sarcoma 180. The effects of this agent on P-388 lymphocytic leukemia cell growth and metabolism are reported.

EXPERIMENTAL

Synthesis—*cis*-Malonato-diammino platinum(II) was synthesized by a two-step procedure (Schemes I and II) utilizing the intermediate diiododiammine platinum(II). The diiododiammine platinum intermediate was synthesized from platinous potassium chloride by the method of Dhara (7). In this procedure, 2.5 mmoles (1 g) of platinous potassium chloride was dissolved in 10 ml of water and filtered until the solution was clear. A saturated solution of potassium iodide (1.65 g in 1.3 ml) was added, and the resulting solution was heated on a steam bath for 5 min, after which 1.4 ml of 3.8 *M* NH_4OH (5 mmoles) was added. The diiododiammine platinum precipitated upon stirring as a caramel-colored powder. This product was cooled in ice, filtered, and washed with hot water, ice-cold ethanol (100%), and ice-cold ether (anhydrous). The yield was 0.925 g.

$$K_2PtCl_4 + 4KI \rightarrow K_2PtI_4 \xrightarrow{NH_4OH} (NH_3)_2PtI_2$$

Scheme I

Finally, *cis*-malonato-diammino platinum(II) was synthesized from diiododiammine platinum by a modification of a reported procedure (8). Diiododiammine platinum(II) (200 mg) was added to 2 ml of a 7% silver nitrate (141 mg) solution. The suspension was heated for 10 min on a steam bath, and the insoluble silver iodide then was removed by filtration on a fritted-glass funnel. The filtrate contained the soluble intermediate

0022-3549/80/1000-1160$01.00/0