Table III—Assay Results on Belladonna Alkaloids on Preparation D Using Procedure 7

Assay	Recovery, %
1	99.7
2	100.3
3	101.2
4	99.2
Average	100.1
Average deviation	±0.65

Procedure 7—Take 25 ml. of the suspension, add 12.5 ml. of concentrated hydrochloric acid, and heat to boiling on a hot plate. Cool, filter, wash the residue with 3 × 10-ml. portions of distilled water, and combine with the filtrate. Adjust the pH of the filtrate to approximately 7.0 with 3.5 *N* sodium hydroxide solution and bring the volume to 100 ml. Take a 5-ml. quantity of this solution and assay according to Procedure 1. The results are presented in Table II.

Preparation D was assayed four times using Procedure 7. The results are presented in Table III.

DISCUSSION

The results of these investigations (Tables I and II) indicate that all three belladonna alkaloids are strongly adsorbed on the surface of magnesium trisilicate. It was impossible to desorb and determine the alkaloids quantitatively with 0.1 *N* hydrochloric acid or other modified procedures (see Procedures 1 through 6). The only method that made them completely available for determination was boiling with concentrated hydrochloric acid. After desorption, the alkaloids could be easily determined using a dye method. As far as various antacids are concerned, the results of these investigations do not indicate adsorption of alkaloids on the surface of aluminum hydroxide gel as reported by Grote and Woods (3). On the other hand, the results are in agreement with those of Blaug and Gross (4) who reported strong adsorption of anticholinergic drugs on the surface of magnesium trisilicate. The method (Procedure 7) is recommended for complete desorption of belladonna alkaloids for analysis. The average assay result on Preparation D was 100.1 ± 0.65 (Table III).

REFERENCES

(1) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 1027.

(2) V. D. Gupta and J. B. Reed, Jr., *Amer. J. Hosp. Pharm.*, **26**, 286(1971).

(3) I. W. Grote and M. Woods, *J. Amer. Pharm. Ass., Sci. Ed.*, **42**, 319(1953).

(4) S. M. Blaug and M. R. Gross, *J. Pharm. Sci.*, **54**, 289(1965).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 3, 1971, from the *College of Pharmacy, University of Houston, Houston, TX 77004*

Accepted for publication April 14, 1972.

Supported by Paul B. Elder Co., Bryan, Ohio, and H. W. Nelson Co., Detroit, Mich.

▲ To whom inquiries should be directed.

Quantitative Determination of D-Dopa Present in Levodopa Samples▲

G. COPPI, A. VIDI, and G. BONARDI

Abstract □ A method for the assay of D-dopa present in L-dopa samples is reported. The method is based on the ability of an L-amino acid decarboxylase, present in a *Streptococcus faecalis* suspension, to convert quantitatively L-dopa to dopamine while D-dopa remains unchanged. The latter is separated from dopamine by an ion-exchange resin and subsequently assayed according to a fluorometric method. Contaminant amounts of D-dopa present in L-dopa samples can be detected.

Keyphrases □ D-Dopa—analysis in levodopa formulations, levodopa biotransformation to dopamine, separation, fluorometry □ Levodopa biotransformation to dopamine—separation, fluorometric analysis of D-dopa □ Dopamine, biotransformation product of levodopa—analysis of D-dopa in levodopa formulations □ L-Amino acid decarboxylase transformation of levodopa to dopamine—analysis of D-dopa in levodopa formulations □ Column chromatography—separation of D-dopa and dopamine □ Fluorometry—analysis, D-dopa

The presence of D-dopa in levodopa (L-dopa) samples, always possible as a result of the synthetic process, is undesirable in the therapy of Parkinson's disease because it has been well established that the D-enantiomer of β-(3,4-dihydroxyphenyl)-α-alanine is biologically inactive and displays toxic side effects (1–3). As a consequence, the quantitative determination of D-dopa in L-dopa samples is of considerable importance. At the moment, a quantitative separation of the two isomers is possible only by liquid chromatography methods (4).

The present authors studied a specific method for determining the contaminant amounts of D-dopa present in L-dopa pharmaceutical preparations. This method is based on the ability of an L-amino acid decarboxylase, present in a *Streptococcus faecalis* suspension, to convert L-dopa into dopamine while D-dopa remains unchanged.

Table I—Determination of D-Dopa in Standard Preparations

Standard Preparations	D-Dopa Found[a] mg.	D-Dopa Found[a] %
L-Dopa, 95.000 mg. + D-dopa, 5.000 mg.	4.950	99.00
L-Dopa, 97.500 mg. + D-dopa, 2.500 mg.	2.570	102.80
L-Dopa, 98.750 mg. + D-dopa, 1.250 mg.	1.310	104.80
L-Dopa, 99.375 mg. + D-dopa, 0.625 mg.	0.619	99.04
L-Dopa, 99.688 mg. + D-dopa, 0.312 mg.	0.304	97.43
		Mean ± *SD* 100.61 ± 1.37

[a] Values represent average of three determinations.

The latter is then separated from dopamine by column chromatography and subsequently assayed fluorometrically.

EXPERIMENTAL

Preparation of *S. faecalis* Suspension—The bacteria used for the enzyme preparation were *S. faecalis* R[1], *S. faecalis* D.A.[2], and *S. faecalis liquefaciens* NCTC 2705. The mentioned strains were grown under anaerobic conditions and maintained by weekly subculture on 2% agar nutrient medium containing 1% glucose, 1% Bacto-casamino acids[3], 0.1% Bacto-yeast extract[3], 0.1% L-tyrosine, and 0.0001% of pyridoxine. The culture was incubated, after inoculation, at 37° for 18–20 hr. and stored at 4°. The *S. faecalis* suspension containing the L-amino acid decarboxylase induced by the presence of L-tyrosine in the nutrient medium was prepared by inoculating the bacteria from the agar culture medium in 10 ml. of the same medium without agar and incubating at 37° for 18–20 hr. The organisms were separated from the medium by centrifugation at 700 *g*, washed two times with sterile physiological saline solution, and finally suspended in a sufficient amount of the same solution to obtain a 10% transmittance at 625 nm. This suspension was immediately used.

Enzymatic Reaction—In a test tube (26 × 110 mm.) fitted with a cap and equipped with tubes (2 mm. i.d.) for the bubbling of nitrogen (2 bubbles/sec.), 3.0 ml. of the *S. faecalis* suspension, 2.0 ml. of L-dopa (2 mg./ml. as a solution in 0.2 *M* acetate buffer, pH 5.5), and 1.0 ml. of pyridoxal 5-phosphate (20 mcg./ml. as a solution in 0.2 *M* acetate buffer, pH 5.5) were added. Nitrogen was used either to obtain anaerobic conditions or to keep the bacteria in suspension. The mixture was incubated in a water bath at 30° for 5 hr. and centrifuged at 70,000 *g* for 30 min., and the supernate was separated.

Separation and Assay of D-Dopa—One milliliter of the supernate was loaded into a column (7 × 40 mm.) containing an ion-exchange resin[4] previously washed with 20 ml. of 2 *N* HCl, 5 ml. of water, 10 ml. of 1 *M* acetate buffer (pH 6.0), and 5 ml. of water. A 0.2-ml./min. volume rate was used for all elution phases. The column was eluted with 0.05 *M* phosphate buffer (pH 6.0). A final volume of 10 ml. eluate was collected. The eluate was used for the fluorometric assay of D-dopa according to Anton and Sayre (5). The residual dopamine was removed from the column by elution with 15 ml. of 2 *N* HCl. The acidic solution was evaporated to dryness *in vacuo*, and the residue was dissolved in 0.5 *M* phosphate buffer (pH 7.0) for the fluorometric assay[5] (5). The excitation and emission spectra were 330 and 380 nm. and 333 and 380 nm. (uncorrected data) for dopa and dopamine, respectively.

Chromatography—Samples of pure dopa and dopamine in comparison with an aliquot (100 μl.) of the supernate derived from the enzymatic reaction were applied on precoated glass plates[6]. The plates were developed with the solvent system of *n*-butanol–acetic acid–water (4:1:5) in a chromatographic chamber oversaturated for 4 hr. The plates were removed after 16 hr., dried, and sprayed with 4% potassium ferricyanide (as a solution in 0.05 *M* phosphate buffer, pH 6.0). The R_f values of dopa and dopamine pure standards were 0.30 and 0.50, respectively.

[1] Sankyo Chemical Industries Ltd.
[2] De Angeli Institute.
[3] Difco.
[4] Amberlite IRC 50.
[5] For the fluorometric assay, a model MPF-2A spectrophotofluorometer (Hitachi, Ltd.) was used.
[6] TLC plates, silica gel F_{254}, Merck.

RESULTS AND DISCUSSION

The induction of an L-amino acid decarboxylase in *S. faecalis* occurred as a result of the incorporation of L-tyrosine in the medium according to the experimental conditions. Probably the induced enzyme is the L-tyrosine decarboxylase (4.1.1.25) which reacts on L-tyrosine and on L-dopa (6). The results of the enzymatic induction were ascertained by determining the enzymatic conversion caused by the organism with both L-dopa and D-dopa and by the subsequent chromatographic assay. For our purposes, a microbiological organism source was considered suitable when it was able to transform quantitatively L-dopa to dopamine without transforming D-dopa after induction with L-tyrosine.

Micrococcus pyogenes var. *aureus* ATCC 6538 P, *Bacillus subtilis* ATCC 6633, *Escherichia coli* McLeod ATCC 10536, *Salmonella typhi* T30 Roma M 507, *Lactobacillus casei* ATCC 7469, and *Lactobacillus arabinosus* ATCC 8014, when assayed according to the same experimental conditions, were inactive. *S. faecalis* (Sup. San. Inst. 1054, Weidbrige 775, Pasteur Inst. 5433, and 2 P, respectively) strains were partially active.

S. faecalis R, *S. faecalis* D.A., and *S. faecalis liquefaciens* NCTC 2705 strains satisfied the requirements and consequently were used interchangeably. When incubated with purified L-dopa (7), these strains transformed it quantitatively to dopamine. The purified D-dopa was not transformed to dopamine. Therefore, with this method it is possible to assay contaminant amounts of D-dopa present in L-dopa samples. It should be emphasized that, in the proposed method, a large amount of dopamine which was enzymatically converted from L-dopa did not interfere with the separation and the assay of D-dopa. According to the experimental conditions, D-dopa recovery from the column was 95 ± 3%.

The good reproducibility of the proposed method is shown in Table I, where the determinations of D-dopa in standard preparations are reported.

REFERENCES

(1) *Brit. Med. J.*, **2**, 202(1969).

(2) G. C. Cotzias, P. S. Papavisiliou, and R. Gellene, *N. Engl. J. Med.*, **280**, 337(1969).

(3) D. C. Poskanzer, *ibid.*, **280**, 383(1969).

(4) R. J. Baczuk, G. K. Landram, R. J. Dubois, and H. C. Dehm, *J. Chromatogr.*, **60**, 351(1971).

(5) A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.*, **145**, 326(1964).

(6) M. Dixon and C. Webb, "Enzimi," Istituto Editoriale Universitario, Milan, Italy, 1971, pp. 627, 740.

(7) A. Brossi, J. E. Heveran, E. A. McMullan, and B. Z. Senkowski, German Offen. 2,003,101 (Aug. 6, 1970); through *Chem. Abstr.*, **73**, 80480b(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 14, 1972, from the *Department of Biochemistry, Research Laboratories of Institute de Angeli, via Serio 15, 20139 Milan, Italy.*

Accepted for publication May 5, 1972.

The authors thank Mr. C. Franco for his technical help and Sankyo Chemical Ind. (Tokyo, Japan) and Welding & Co. (Amburgo) for supplying some of the materials and information mentioned.

▲ Inquiries should be directed to: Prof. Silvano Casadio, Institute de Angeli, via Serio 15-P.O.B. 3197, Milan, Italy.