to ragweed pollen and Ammi tissue culture. The response to the latter was low (5×10^2 units/mg of specific activity). Plant extract fractions generally elicited a stronger allergenic reaction than tissue culture fractions.

Of all of the fractions tested, the pollen extract consistently gave the highest allergenic activity. Ragweed-sensitive patients (S.R. and T.M.) reacted consistently to the plant materials and tissue cultures tested, whereas ragweed-nonsensitive individuals did not.

Immunodiffusion and Electrophoresis—Immunodiffusion analysis was performed to observe if Fraction D from plants and tissue cultures cross-reacted with short ragweed pollen and ragweed antigen E. No precipitin lines were observed between plant and tissue culture Fraction D and anti-antigen E serum (Fig. 1). None of the fractions tested, except ragweed pollen, contained allergens that have a total identity or partial identity with antigen E. Several fractions from short ragweed tissue cultures, Ammi tissue culture, marigold tissue culture, and marigold plant (weak) did have precipitin lines against anti-short-ragweed-pollen serum. It is possible that those fractions contained either other ragweed allergens, such as antigen K, Ra_3, or common proteins, *e.g.*, enzymes. It is presently unknown if the hypersensitivity reactions observed are specific reactions to Fraction D or a general hypersensitivity toward proteins that is peculiarly demonstrated by ragweed-sensitive patients. There also is some evidence that Fraction C from both plants and tissue cultures may contain allergens.

Polyacrylamide gel electrophoresis was used to determine if the plant and tissue culture allergenic fractions contained proteins with similar electrophoretic characteristics as antigen E. All plant and tissue culture fractions had a protein band in the antigen E region (Fig. 2). Ammi tissue culture, cantaloupe tisue culture, and short ragweed tissue culture had strong bands. The proteins in this region are responsible for the high allergenic activity in skin testing. However, further work is required to examine the proteins and allergenic properties of the protein band observed.

REFERENCES

(1) T. P. King and P. S. Norman, *Biochemistry*, **1**, 709(1962).

(2) T. P. King, P. S. Norman, and J. T. Connel, *ibid.*, **6**, 1992(1964).

(3) T. P. King, P. S. Norman, and L. M. Lichtenstein, *ibid.*, **3**, 458(1967).

(4) A. Shafiee and E. J. Staba, *In Vitro*, **9**, 19(1973).

(5) B. Anderson, E. M. Dreyfuss, J. Logan, D. E. Johnston, and J. Glaser, *J. Allergy*, **45**, 310(1970).

(6) E. J. Staba, in "Recent Advances in Phytochemistry," vol. 2, M. K. Seikel and V. C. Runeckles, Eds., Appleton-Century-Crofts, New York, N.Y., 1969, pp. 75–106.

(7) O. H. Lowry, N. J. Rosenberg, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265(1951).

(8) A. Shafiee, E. J. Staba, and Y. J. Abul-Hajj, *J. Pharm. Sci.*, **62**, 1654(1973).

(9) O. Ouchterlony, *Progr. Allergy*, **5**, 1(1958).

(10) C. A. Williams and M. W. Chase, in "Methods in Immunology and Immunochemistry," vol. 3, C. A. Williams and M. W. Chase, Eds., Academic, New York, N.Y., 1971, pp. 321–339.

(11) B. J. Davis and L. Ornstein, in "Methods in Immunology and Immunochemistry," vol. 2, C. A. Williams and M. W. Chase, Eds., Academic, New York, N.Y., 1968, pp. 38–47.

(12) L. Berrens, "The Chemistry of Atopic Allergens," S. Karger, New York, N.Y., 1971, pp. 6, 7.

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Single-Tablet Enantiomeric Purity Assay of Amphetamine by Rotation Enhancement

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Abstract □ Enhancement of the optical rotation of dextroamphetamine by production of an optically active chromophore through reaction with 1-fluoro-2,4-dinitrobenzene to give α-methyl-*N*-(2,4-dinitrophenyl)-β-phenylethylamine is reported. This reaction forms the basis of an assay for both the content and optical purity of dextroamphetamine sulfate at single-dose levels (5 mg). Results of assays of standard solutions and of commercial tablets demonstrate the suitability of the method for the determination of enantiomeric purity and amphetamine content.

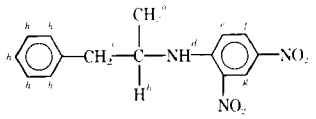
Keyphrases □ Amphetamine—colorimetric analysis, optical purity assay, commercial tablets □ Colorimetry—analysis, amphetamine, commercial tablets

The USP XIX (1) isomeric purity[1] test for dextroamphetamine sulfate (I) requires separation of the amphetamine from dosage excipients and conversion to *N*-acetylamphetamine, followed by isolation, purification, and determination of the specific rotation of the derivative. This method suffers from low sensitivity; 130 mg of the drug is specified for the test, although amphetamine sulfate is commonly formulated in 5- or 10-mg dosage units. Some very sensitive tests (2, 3), which depend on physical examination of crystalline derivatives, readily distinguish *d*- from *dl*- and *l*-amphetamines at milligram levels, but they cannot be used for the determination of enantiomeric purity[1].

Although several NMR methods for the determination of enantiomeric composition were reported re-

[1] Isomeric purity, also called enantiomeric purity, is a measure of the percent of one enantiomer. Optical purity is the percent of the specific rotation of the pure enantiomer. The relationship between them is given in Eqs. 2 and 3.

Table I—NMR Data for Compound III



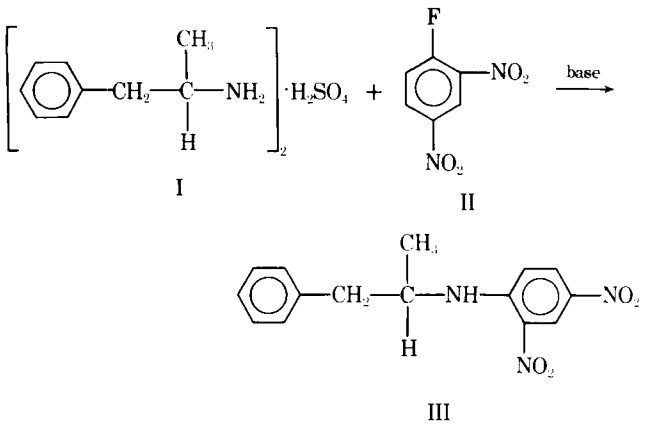
Proton	Chemical Shift[a], ppm δ	Coupling
a	1.38 (d)	J_{ab} = 7 Hz
b	4.05 (sext)	$J_{ab} = J_{bc}$ = 7 Hz
c	3.95 (d)	J_{bc} = 7 Hz
d	8.6 (s) broad	—
e	6.85 (d)	J_{ef} = 10 Hz
f	8.15 (d, d)	J_{ef} = 10 Hz, J_{fg} = 3 Hz
g	9.25 (d)	J_{fg} = 3 Hz
h	7.25 (s)	—

[a] (s) = singlet, (d) = doublet, (d, d) = doublet of doublets, (sext) = sextet.

cently (4–6), none of these has been developed into a working assay. A GLC method for the separation of enantiomers of amphetamine (7) has been developed into a working assay (8), collaboratively studied (9), and adopted as an official assay by the Association of Official Analytical Chemists (10). The method is sufficiently sensitive for a single-tablet assay but requires considerable manipulation; problems with instability of the reagent were also reported (7–9).

This report describes the reaction of I with 1-fluoro-2,4-dinitrobenzene (II) to form the 2,4-dinitrophenyl derivative, α-methyl-*N*-(2,4-dinitrophenyl)-β-phenylethylamine (III) (Scheme I). This derivative has an optical rotation at 546 nm, which is about 30 times greater than that of I itself. In acid solution, the absorptivity of III at 430 nm is linearly related to the concentration of I and is free from interferences from the reagent and by-products. This colorimetric reaction forms the basis for the determination of both the amphetamine content and apparent specific rotation[2] of III. These two measurements on separate dilutions of the same reaction mixture are used to calculate the amount of I and its optical purity[1].

No prior isolation is required, but the sample must be known to be free of primary or secondary amines. Reagents are added directly to the ground tablet; the



I II III

Scheme I

[2] Apparent specific rotation because the weight of I is used in the calculation, but rotation of III is measured.

Table II—Percent I Found *versus* Enantiomeric Composition of Amphetamine Sulfate

Composition Prepared as Percent I[a]	I Found, %	
100	99.1	98.9
89.9	91.2	91.2
79.9	78.9	79.1
69.9	71.4	74.8
59.9	60.7	—
50[b]	50.1	51.3
39.4	42.2	41.7

[a] Mixture was prepared by weighing samples of I and levoamphetamine sulfate into a volumetric flask; two aliquots were assayed. [b] Racemic amphetamine sulfate was used.

product, III, along with excess reagents, is separated from tablet excipients by column partition chromatography. The eluate is adjusted to a suitable concentration and measured polarimetrically and spectrophotometrically.

The use of reagent II for end-group analysis of proteins (11), gravimetric determination of phenols (12), and microanalysis of primary amines (13) is well known. However, its use in rotation enhancement appears to be novel.

EXPERIMENTAL

Instruments and Apparatus—An automatic polarimeter[3] capable of measuring rotations at 546 nm with a precision of ±0.005°, a spectrophotometer[4], a spectropolarimeter[5], and an NMR spectrometer[6] were used. Chromatographic tubes[7], 22 mm i.d. × 250 mm long, with a 6-mm i.d. tube affixed to the base, a tamping rod consisting of a metal disk about 21 mm in diameter on a rod about 600 mm long, and conventional glassware are required. A rotary evaporator[8] also was used.

Preparation of Compound III—Dextroamphetamine base[9] (2 ml), II (0.5 ml), and triethylamine (0.5 ml) were dissolved in 20 ml of dioxane, and the mixture was allowed to stand at room temperature. The reaction was monitored by TLC[10] until all II was consumed (about 2 hr). Dioxane and some excess amphetamine were removed under reduced pressure on a rotary evaporator.

The residue was dissolved in 100 ml of chloroform and passed over a chromatographic column packed with 10 ml of 4 *N* H_2SO_4 on 25 g of diatomaceous earth[11]. The column was eluted with an additional 100 ml of chloroform, and the combined chloroform eluate was evaporated on the rotary evaporator, leaving a residual yellow oil.

About 50 mg of the residue, Compound III, was dissolved in 0.5 ml of deuterated chloroform, dried with about 100 mg of anhydrous magnesium sulfate, and filtered into a 5-mm NMR tube; then the NMR spectrum was obtained (Table I). About 1 mg of III was dissolved in 10 ml of methanol, and the optical rotatory dispersion curve was determined in a 1-mm cell from 600 to about 380 nm, below which absorption became too great for further measurement of the rotation (Fig. 1).

[3] Perkin-Elmer model 141M automatic polarimeter.

[4] A Cary model 15 spectrophotometer was used in the time scan mode; thus, all measurements could be made without changing the monochromator settings since the absorption at 430 nm is on a slope.

[5] Model JASCO-ORD/CD-5, Japan Spectroscopic Co.

[6] Varian A-60 analytical spectrometer.

[7] Kontes Glass Catalog No. K 420300.

[8] Buchi Rotavapor-R rotary evaporator.

[9] Aldrich Chemical Co. ($[\alpha]_D$ + 34.0°, *c* = 10 in methanol, n_D^{20} 1.5170). It was used without further purification.

[10] Silica gel GF, 250 μm thick on 25 × 75-mm microscope slides, was spotted with 5 μl of reaction mixture, dried, and developed with chloroform. The product, III, was observed as a yellow spot under white light (R_f 0.75), and both III and II (R_f 0.50) were observed as dark spots under long wavelength UV. Excess I and triethylamine and their hydrofluorides were at the solvent front.

[11] Celite 545, acid washed, Johns-Manville Corp.

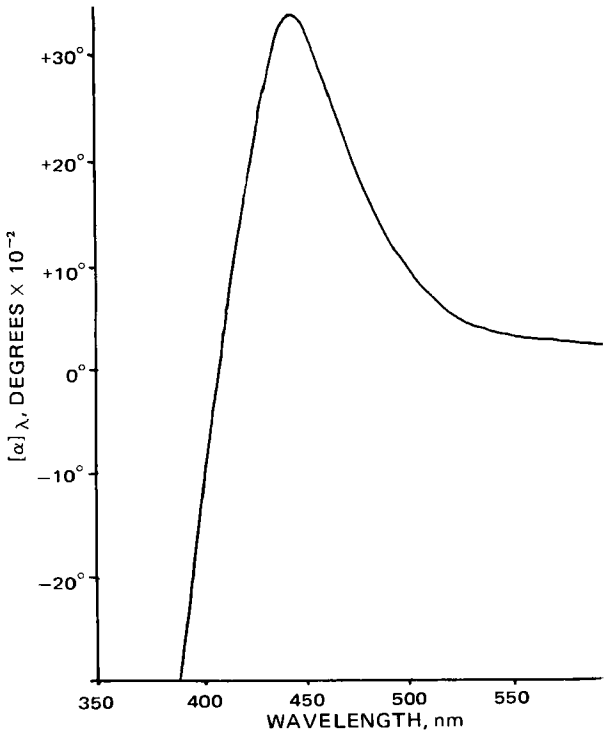


Figure 1—*Optical rotatory dispersion curve of III in methanol. Specific rotations are approximate, estimated from the known value at 546 nm.*

Reagents—The following were used: diatomaceous earth[11], acid-washed; 2% II in tetrahydrofuran; 10% sodium carbonate solution; 10% sodium citrate solution; diluted hydrochloric acid (1:3); water-saturated chloroform; dimethyl sulfoxide; and methanol.

Standard Solution—Dissolve sufficient USP dextroamphetamine sulfate reference standard, accurately weighed, in water to prepare a solution containing, in each 2 ml, an amount equivalent to the content of the tablet under analysis.

Procedure—Prepare separate columns for sample, standard, and blank. Thoroughly mix 3 g of diatomaceous earth and 1 ml of 10% sodium carbonate solution and transfer to chromatographic tubes, each of which has a wad of glass wool[12] in the base for support. Pack each column to a uniform mass with the tamping rod and position each column to elute into a 250-ml erlenmeyer flask.

Place single tablets or weighed amounts of composite equivalent to one tablet in individual 150-ml beakers. Add 2 ml of water to each beaker and to a separate beaker to serve as a blank. To another beaker, add 2.0 ml of standard solution. Gently agitate until the tablets completely disintegrate; then add 1 ml of dimethyl sulfoxide to each beaker. Mix by gentle agitation and add 5 ml of 2% II in tetrahydrofuran, followed by 1 ml of 10% sodium citrate solution. Let stand for 20 min with occasional gentle agitation, then add 5 g of diatomaceous earth to each beaker, and mix thoroughly. Add 50 ml of water-saturated chloroform, suspend the diatomaceous earth by stirring, and transfer to the column with the aid of 10 ml of chloroform, collecting the eluate in the 250-ml erlenmeyer flask. Add 1 g of diatomaceous earth and a few drops of water to the beaker and scrub the beaker, mixing the diatomaceous earth until it is uniform in texture and color.

After the column has drained, pack with the tamping rod, transfer the "dry wash" from the beaker, tamp, and top the column with a wad of glass wool. Elute the column with two 50-ml portions of water-saturated chloroform, collecting the combined eluates in the 250-ml flask. Evaporate the chloroform on a steam bath under a stream of air until only a viscous yellow liquid remains. Then dissolve the residue in 2 ml of methanol and, with the aid of a long-tip disposable pipet, transfer the solution to a 10-ml volumetric flask. Repeat with successive 2-ml portions of methanol, washing down the walls of the flask to effect quantitative transfer, and dilute to volume with methanol.

[12] Pyrex Filtering Fibre, Corning Glass Catalog No. 3959.

Table III—Standard Recoveries of I and Enantiomeric Purity of I

	Apparent[a] $[\alpha]_{546}$	Recovery[b], %	Enantiomeric Purity[c], %
	+788°	99.2	99.9
	+794°	97.5	100.3
	+792°[d]	99.7	100.2
	+779°[d]	98.1	99.4
	+786°[e]	100.5	99.8
	+790°[e]	99.7	100.0
	+794°	103.6	100.3
	+790°	102.5	100.0
	+792°	99.2	100.2
Average	+789°	100.0	100.0
SD	±5°	±1.96	±0.29
Coefficient of variation	±0.597	±1.96	±0.29

[a] See footnote 2 in text. [b] Percent of average value of absorptivity. [c] Average value of $[\alpha]_{546}$ was used as 100% reference. [d] One hundred milligrams of starch was added to an aliquot of the standard solution. [e] Three hundred milligrams of starch was added to an aliquot of the standard solution.

Transfer to a 1-dm cell and measure the rotation at 546 nm. Transfer 3.0 ml of the solution to the 100-ml volumetric flask containing 10 ml of diluted hydrochloric acid and adjust to volume with methanol. Measure the absorption of this solution in 1-cm cells at 430 nm *versus* the blank treated in the same manner. Similarly, measure the rotation at 546 nm and absorbance at 430 nm of the procedural standard.

From the absorbance of the sample and standard at 430 nm, calculate the concentration, c, of amphetamine sulfate in grams per 100 ml in the sample taken. Use this value in the calculation of specific rotation. From the absorbance of the sample and standard and the exact weight of the standard, calculate the apparent specific rotation, $[\alpha]_{546}$, of the standard and samples from:

$$[\alpha]_{546} = \frac{100\alpha}{lc} \qquad \text{(Eq. 1)}$$

where c is the concentration, in grams per 100 ml, of amphetamine sulfate calculated for the samples (or taken for the standard); α is the measured rotation in degrees; and l is the length of the polarimeter cell in decimeters (1 dm in this case).

Calculate the optical purity from the apparent specific rotations and:

$$\text{optical purity} = \frac{[\alpha_u]_{546}}{[\alpha_s]_{546}} \times 100 \qquad \text{(Eq. 2)}$$

where u and s refer to the sample and standard, respectively. The enantiomeric purity is calculated from:

$$\text{enantiomeric purity} = \frac{(\text{optical purity})}{2} + 50 \qquad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

The product of the reaction, III, was characterized by its NMR spectrum; the data given in Table I are consistent with the assigned structure. The optical rotatory dispersion curve shown in Fig. 1 has a large maximum at about 440 nm. The rotation is not measured at this wavelength, because the ratio of molar absorptivity to molar rotation of III is large at 440 nm and a solution sufficiently concentrated to give a measurable rotation is almost opaque even in a 0.01-dm cell. Because of instrumental limitations, rotations were measured at 546 nm, rather than the preferable wavelength at about 500 nm, where the apparent specific rotation is about 200% greater. The automatic polarimeter uses discharge lamps as its light source, and the intense mercury emission line nearest 440 nm at which the absorption of III will not interfere is at 546 nm.

The rotation of the reaction mixture showed a linear relationship to enantiomeric composition from 0 to 100% optical purity. The data for samples containing 40 to 100% of I (the balance was

Table IV—Enantiomeric Purity and Content of I in Commercial Tablets[a]

Milligrams per Tablet Found	Percent of Declared	$[\alpha]_{546}$[b]	Enantiomeric Purity, %
4.65	92.9	+656°	92.4
4.59	91.9	+662°	92.8
4.65	92.9	+677°	93.8
4.44	88.9	+674°	93.5
Average	91.6		93.1
SD	±1.89		±0.63
Coefficient of variation	±2.07		±0.68

[a] Content and enantiomeric purity were determined on the same sample as described in text. [b] The $[\alpha]_{546}$ of the standard was +773°.

levoamphetamine sulfate[13]) are given in Table II. The exact specific rotation of III was not determined and the apparent specific rotation, based on weight of I, varied slightly with reaction conditions. The problem of the variation of the apparent specific rotation was resolved by the use of a procedural standard run concomitantly with the samples.

The reaction between I and II takes place readily under a variety of conditions in a variety of solvents, particularly alcohols. The reaction conditions described were selected to provide for the facile separation of dyes and other tablet excipients that would interfere with the spectrophotometric measurement. Dimethyl sulfoxide was used to promote solubilization (14) because results were 2–5% low on tablet composites without it. The columns were wet-packed because tetrahydrofuran stripped the trap layer during elution when the diatomaceous earth–reaction mixture was packed on the column directly.

The results of the assay of standard solutions with 300, 100, and 0 mg of starch added are shown in Table III. Values of 100.0 ± 0.29% (*SD*) for enantiomeric purity and 100.0 ± 1.96% for percent amphetamine sulfate were obtained. The results with commercial tablet samples, 93.1 ± 0.63% for enantiomeric purity and 91.6 ± 1.87% for amphetamine sulfate content (Table IV), are in accord with a value of 93.5 ± 1.58% for amphetamine sulfate content obtained by an independent column chromatographic–spectrophotometric assay[14]. In every case, there was a distinct improvement in the precision of the enantiomeric purity calculated.

The marked improvement of the standard deviation for enantiomeric purity over that of amphetamine content was due in part to the method of calculation, but half of the improvement was real. The reason for the improvement is that the optical rotation and absorptivity were determined on the same sample in each case and were then used to calculate specific rotation. This method seems to eliminate a random, although unidentified, error which affects both optical rotation and amphetamine content. The improvement in precision of enantiomeric purity *vis-à-vis* specific rotation is only apparent, the result of the calculation.

[13] Levoamphetamine sulfate, K & K Laboratories ($[\alpha]_D$ − 21.0°, c = 10 in water). Free base was isolated, its (−)-tartrate was recrystallized three times from alcohol, and the recovered base was converted to the sulfate by an unpublished method of L. H. Welsh to give $[\alpha]_D$ − 23.4°, c = 10 in water.

[14] The assay used is an unpublished one, developed and validated by T. D. Doyle of this laboratory.

REFERENCES

(1) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975.

(2) J. H. Clark, *J. Ass. Offic. Anal. Chem.*, **56**, 1065(1973).

(3) J. A. Heagy, *Anal. Chem.*, **42**, 1459(1970).

(4) W. H. Pirkle, S. D. Beare, and T. G. Burlingame, *J. Org. Chem.*, **34**, 470(1969).

(5) J. A. Dale, D. L. Dull, and H. S. Mosher, *ibid.*, **34**, 2543(1969).

(6) G. M. Whiteside and D. W. Lewis, *J. Amer. Chem. Soc.*, **92**, 6979(1970).

(7) E. Gordis, *Biochem. Pharmacol.*, **15**, 2124(1966).

(8) C. E. Wells, *J. Ass. Offic. Anal. Chem.*, **53**, 113(1970).

(9) *Ibid.*, **55**, 146(1972).

(10) A. Romano, *J. Ass. Offic. Anal. Chem.*, **55**, 252(1972).

(11) F. Sanger, *Biochem. J.*, **39**, 507(1945), *et seq.*

(12) P. A. Lehmann, *Anal. Chim. Acta*, **54**, 321(1971).

(13) F. C. McIntire, L. M. Clements, and M. Sproull, *Anal. Chem.*, **25**, 1757(1953).

(14) J. Levine and T. D. Doyle, *J. Pharm. Sci.*, **56**, 619(1967).

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