(2) C. Hansch, J. E. Quinlan, and G. L. Lawrence *J. Org. Chem.*, **33**, 347 (1968).
(3) W. O. M. McReynolds, "Gas Chromatographic Retention Data," Preston Technical Abstracts Co., Evanston, Ill., 1964.
(4) S. Wold and K. Andersson, *J. Chromatogr.*, **80**, 43 (1973).
(5) D. L. Massart, P. Lenders, and M. Lauwereys, *J. Chromatogr. Sci.*, **12**, 617 (1974).

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Fluorometric Determination of Methyldopa in Biological Fluids

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Abstract □ A fluorometric method for the analysis of methyldopa, based on the formation of a fluorophore after oxidation and rearrangement, is described. The drug is isolated from biological fluids by adsorption on alumina and elution with an organic solvent. Fluorescence is linear from 0.1 to 1.5 μg of methyldopa/ml. The assay has a lower limit of sensitivity of 100 ng/ml and is suitable for pharmacokinetic studies following therapeutic doses in animals and humans.

Keyphrases □ Methyldopa—fluorometric analysis, biological fluids □ Fluorometry—analysis, methyldopa in biological fluids □ Antihypertensive agents—methyldopa, fluorometric analysis in biological fluids

Methyldopa is a widely used antihypertensive agent with sedative and aromatic L-aminodecarboxylase-inhibiting properties (1–7). Methods to analyze the drug in pharmaceutical preparations and biological fluids have utilized visible spectrophotometry (8, 9), GLC (10), high-pressure liquid chromatography (11), and fluorometry (12, 13). In addition, tracer methodology has been used for metabolic and disposition studies (14, 15).

Fluorometric methods have been primarily based on the production of a fluorescent indole derivative (lutin) by oxidation and subsequent rearrangement. This technique has also been applied to estimate catecholamines and related compounds (16). Existing fluorometric methods appear to be insufficiently sensitive for the determination of drug levels in biological fluids.

This paper describes an improved analytical method in which methyldopa is adsorbed on alumina, eluted with an organic solvent, and then oxidized to form the fluorophore, dihydroxyindole. The establishment of optimal conditions for maximal fluorescence resulted in an increased sensitivity for estimating methyldopa in biological fluids. The method has provided increased sensitivity, consistency, and reproducibility.

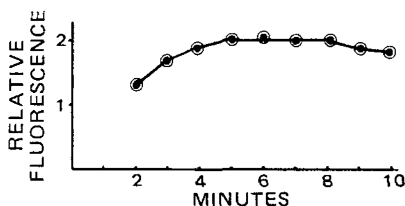

Figure 1—*Stability of methyldopa oxidation product.*

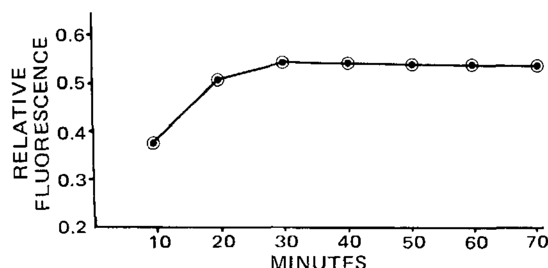

Figure 2—*Stability of fluorophore of methyldopa following oxidation and rearrangement.*

EXPERIMENTAL

Apparatus—All fluorescence measurements were made using a spectrophotofluorometer[1]. Microphotometer sensitivity was set at 30 with multiplier positions ranging from 0.01 to 0.1.

Reagents—All chemicals and solvents were analytical reagent grades, and freshly distilled water was used. All glassware was rinsed with 10% nitric acid and distilled water. Alumina[2] was reactivated by refluxing in 2 *N* HCl, washing with distilled water until the pH was 3.5–4, and drying at 250–300°. Methyldopa was used as received[3].

The phosphate buffer was prepared by adding 0.1 *M* dibasic sodium phosphate containing 0.1% edetate sodium to 0.1 *M* monobasic sodium phosphate containing 0.1% edetate sodium.

Analytical Procedure—Blood samples were collected in heparinized tubes containing 15 mg of sodium metabisulfite. The blood was centrifuged immediately, and the plasma was separated and frozen until assayed. Aliquots of biological fluids (0.1–1.0 ml) were placed in screw-capped test tubes, and 4–5 ml of 0.4 *N* perchloric acid was added. After shaking for 5 min, the precipitated protein was separated by centrifugation.

A specific volume (1–5 ml) of the supernate was transferred to a screw-capped test tube containing 0.5 g of activated alumina and 0.25 g of edetate sodium. The mixture was adjusted to pH 8.5–8.6 with 1 *N* sodium carbonate with constant stirring, and shaking was continued for an additional 5 min. The alumina was allowed to settle, and then the supernate was aspirated and discarded. The alumina was washed twice with 5 ml of distilled water, and the washings were discarded. The adsorbed methyldopa was eluted by vigorously shaking the washed alumina for 15 min with 6 ml of acetone–formic acid (85:15).

The alumina suspension was centrifuged, and 5.0 ml of the supernate was transferred to a test tube and evaporated under a nitrogen stream at 40°. The dried residue was dissolved in 1 ml of pH 6.5 phosphate buffer. Then 0.1 ml of ethanol, 0.05 ml of 0.2% zinc sulfate, and 0.05 ml of 0.1%

[1] Aminco-Bowman, American Instrument Co.
[2] Woelm Neutral Activity Grade 1.
[3] Courtesy of Merck Sharp & Dohme Laboratories.

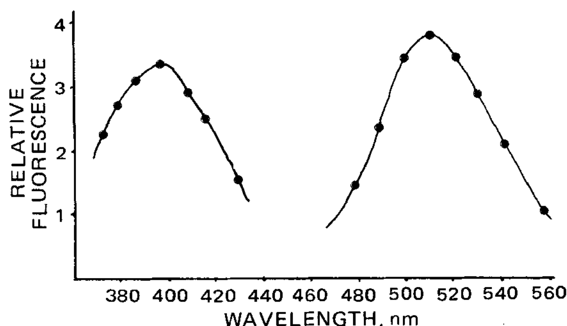

Figure 3—*Wavelength maxima for excitation (left) and emission (right) of methyldopa fluorophore.*

potassium ferricyanide were added, and the mixture, after shaking, was allowed to stand for 6 min at room temperature. After the reaction, 0.5 ml of 5.0 *N* NaOH–1% ascorbic acid (9:1) was added, and the solution was allowed to stand at room temperature for an additional 40 min. The fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 510 nm.

Plasma samples with added known amounts of methyldopa were analyzed, and a standard curve of fluorescence *versus* concentration was determined. Control plasma and urine samples were analyzed to assess background fluorescence arising from fluorescent materials in biological fluids. Other commonly used antihypertensive agents such as guanethidine, hydralazine, propranolol, polythiazide, and reserpine were added to methyldopa samples to determine interference.

RESULTS AND DISCUSSION

Methyldopa is selectively adsorbed on activated alumina and is eluted under acidic conditions. Catecholamines have similar characteristics, but the methyldopa concentration under therapeutic conditions was usually many hundredfold greater than the endogenous catecholamine levels. The major metabolites of methyldopa, 3-*O*-methylmethyldopa and methyldopa *O*-sulfate, were not adsorbed on alumina at pH 8.5–8.6 and thus were separable from the parent compound (17, 18).

Anton and Sayre (19) studied factors affecting alumina adsorption of trihydroxyindole for the analysis of catecholamines and reported that pH adjustment of sample solutions containing alumina and edetate sodium and the pH of the eluting solvent were critical variables. In previously reported catecholamine assays, sodium hydroxide and potassium carbonate solutions were used for the adjustment of sample solution pH (19, 20). Methyldopa is rapidly destroyed in solutions of high pH, and the recovery is poor when using sodium hydroxide. Potassium carbonate precipitates perchloric acid from solution as potassium perchlorate, with some drug coprecipitating. Sodium carbonate solution gave improved results.

Previously reported methods used 0.01 *N* $HClO_4$ and 0.5 *N* acetic acid to elute the adsorbed methyldopa from alumina (19, 21, 22). The method reported here, with an acetone–formic acid (85:15) mixture, gave improved recovery of adsorbed drug. Plasma samples spiked with 0.1, 0.5, and 1.0 μg of methyldopa gave average recoveries (five determinations) of 83.3 ± 2.7, 86.0 ± 1.6, and 91.6 ± 1.4%, respectively.

The oxidation of methyldopa to a chromophoric species was accomplished using potassium ferricyanide at pH 6.5. This oxidation was further improved by the addition of 0.1 ml of ethanol and 0.05 ml of 0.2% zinc sulfate.

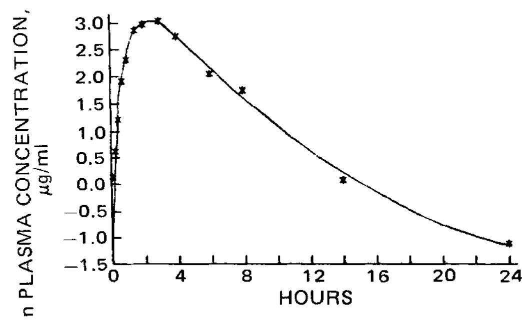

Figure 4—*Computer-drawn (SPEAKEZ) plasma level–time profile following a 300-mg oral dose of methyldopa in a rabbit.*

Table I—Analysis of Methyldopa in the Presence of Other Antihypertensive Drugs

Mixture[a]	Fluorescence[b], Arbitrary Units
Methyldopa	6.2 ± 0.3
Methyldopa–guanethidine sulfate	6.3 ± 0.2
Methyldopa–hydralazine	6.1 ± 0.4
Methyldopa–propranolol	6.0 ± 0.3
Methyldopa–polythiazide–reserpine	6.1 ± 0.2

[a] Methyldopa, 5 μg, and 20 mg of other drugs except 2 μg of polythiazide and 0.25 μg of reserpine. [b] Mean of four assays ± *SD*.

The oxidation reaction was completed within 5 min, and the product was stable up to 8 min (Fig. 1). In the reported method, 6 min was chosen as the oxidation reaction time. The formation of the fluorophore was completed within 40 min after the addition of ascorbic acid–sodium hydroxide and was stable up to 70 min (Fig. 2). The wavelength maxima of excitation and emission were 400 and 510 nm, respectively (Fig. 3). The standard curve of relative fluorescence *versus* concentration was linear over the concentration range of 0.1–1.5 μg/ml. The lower limit of sensitivity was 100 ng/ml. At this concentration, the sample fluorescence was at least twice that of the background. Below this concentration, the reproducibility of the analytical method was variable.

The applicability of the assay to clinical studies was demonstrated by determining the plasma concentration–time curve of methyldopa in a fasted rabbit after a single oral dose of 300 mg of methyldopa. The results were treated by a SPEAKEZ nonlinear computer program (Fig. 4). Urine samples were collected for 24 hr and assayed; 91.2 mg of free drug (30%) was excreted in 24 hr.

Experiments in which methyldopa was not separated from plasma or urine showed significant interference. Other widely used antihypertensive agents (*e.g.*, guanethidine sulfate, hydralazine, propranolol, polythiazide, and reserpine) did not interfere in this assay (Table I). This fluorescence method is rapid and simple for the determination of methyldopa in biological fluids and is applicable for pharmacokinetic studies in animals and humans.

REFERENCES

(1) J. A. Oates, L. Gillespie, S. Udenfriend, and A. Sjoerdsma, *Science*, **131**, 1890 (1960).

(2) C. T. Dollery and M. Harington, *Lancet*, **1**, 759 (1962).

(3) P. J. Cannon, R. T. Whitlock, C. R. Morris, M. Angers, and J. H. Laragh, *J. Am. Med. Assoc.*, **179**, 673 (1962).

(4) L. Gillespie, J. A. Oates, J. R. Crout, and A. Sjoerdsma, *Circulation*, **25**, 281 (1962).

(5) R. O. H. Irvine, K. P. O'Brien, and J. D. K. North, *Lancet*, **1**, 300 (1962).

(6) H. Smirk, *Br. Med. J.*, **1**, 146 (1963).

(7) N. Nikos, C. Victor, and F. A. Finnerty, *Am. J. Cardiol.*, **13**, 489 (1964).

(8) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, pp. 319, 320.

(9) S. K. Wahba Khalil and R. B. Salama, *J. Pharm. Pharmacol.*, **26**, 972 (1974).

(10) J. R. Watson and R. C. Lawrence, *J. Chromatogr.*, **103**, 63 (1975).

(11) P. D. Walson, K. S. Marshall, R. P. Forsyth, R. Rapoport, K. L. Melmon, and N. Castagoli, Jr., *J. Pharmacol. Exp. Ther.*, **195**, 1 (1975).

(12) T. L. Sourkes, G. F. Murphy, and B. C. Lara, *Br. J. Med. Pharm. Chem.*, **5**, 204 (1962).

(13) A. Sjoerdsma, A. Vendsalu, and K. Engelman, *Circulation*, **28**, 492 (1963).

(14) R. P. Buhs, J. L. Beck, O. C. Speth, J. L. Smith, N. R. Trenner, P. J. Cannon, and J. H. Laragh, *J. Pharmacol. Exp. Ther.*, **143**, 205 (1964).

(15) L. F. Prescott, R. P. Buhs, J. O. Beattie, O. C. Speth, N. R. Trenner, and L. Lasagna, *Circulation*, **34**, 308 (1966).

(16) R. Laverty and K. M. Taylor, *Anal. Biochem.*, **22**, 269 (1968).

(17) J. R. Bianchine, L. R. Calimlim, J. P. Morgan, C. A. Dujuvne, and L. Lasagna, *Ann. N.Y. Acad. Sci.*, **179**, 126 (1975).

(18) R. D. Hoeldtke and J. W. Sloan, *J. Lab. Clin. Med.*, **75**, 159 (1970).

(19) A. H. Anton and D. F. Sayre, *J. Pharmacol.*, **138,** 360 (1962).
(20) A. Bertler, A. Carlsson, and E. Rosengren, *Acta Physiol. Scand.*, **44,** 273 (1958).
(21) A. H. Anton and D. F. Sayre, *J. Pharmacol.*, **145,** 326 (1964).
(22) T. L. Sourkes and G. F. Murphy, "Methods in Medicinal Research," vol. IX, Medical Publisher, Chicago, Ill., 1961, pp. 147–152.

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GLC Determination of *N,N*-Dimethylaniline in Penicillins

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Abstract ☐ A reliable GLC procedure was developed for the determination of residual *N,N*-dimethylaniline as a contaminant in ampicillin commercial samples from various sources. This procedure was similarly applied to other penicillins and cephalosporins. The method involves dissolution of the sample in aqueous alkali, extraction of the organic base with cyclohexane containing naphthalene as an internal standard, and injection into a gas chromatograph with a phenyl methyl silicone column. Levels of 0.1 ppm of dimethylaniline were easily measured with a coefficient of variation less than 10%, and recoveries from spiked samples exceeded 99%.

Keyphrases ☐ *N,N*-Dimethylaniline—GLC analysis in various penicillins and cephalosporins, commercial samples ☐ Penicillins, various—GLC analysis of *N,N*-dimethylaniline as contaminant in commercial samples ☐ Cephalosporins, various—GLC analysis of *N,N*-dimethylaniline as contaminant in commercial samples ☐ GLC—analysis, *N,N*-dimethylaniline in various penicillins and cephalosporins, commercial samples ☐ Contaminants—*N,N*-dimethylaniline, GLC analysis in various penicillins and cephalosporins, commercial samples ☐ Antibacterials—various penicillins and cephalosporins, GLC analysis of *N,N*-dimethylaniline as contaminant in commercial samples

The premarketing certification process assures that each bulk and dosage form batch of antibiotics intended for human use complies with the specifications of proposed and established standards for identity, potency, quality, and purity.

The Code of Federal Regulations provides for Good Manufacturing Practices (GMP) in the production of pharmaceuticals. The direction to "... minimize contamination of products by extraneous adulterants..." (1) applies also to residual reagents that may exhibit undesirable properties such as toxicity or carcinogenicity during antibiotic therapy and/or possible accumulation from other drugs.

Ampicillin, a semisynthetic penicillin, has been synthesized through diverse routes (2) by using 6-aminopenicillanic acid (II) as an intermediate and an organic base such as *N,N*-dimethylaniline (I) to abstract generated hydrogen chloride, the presence of which inhibits the synthesis.

Although gross contaminations can normally be detected by the general analytical techniques employed in certification, trace impurities are often too elusive. As the Food and Drug Administration became increasingly aware of the adventitious presence of residual dimethylaniline in ampicillin, the GLC method was developed to ascertain the extent of the problem. The pharmacological properties of dimethylaniline have not been fully elucidated, but it seemed advisable to limit its presence because of its dubious nature and possible carcinogenicity.

This report presents and discusses a GLC method[1] for the analysis of residual dimethylaniline in ampicillin and its applicability to survey-related antibiotics.

EXPERIMENTAL

Apparatus—A gas chromatograph[2] was fitted with an inlet system modified to accommodate a one-piece glass column directly from the site of injection, as described previously (3), and with a flame-ionization detector. It was used together with a 1-mv range strip-chart recorder. The instrument was also equipped with individual controls, allowing for separate heating of the inlet, column, and outlet. Peak area measurements were made with a digital electronic integrator[3].

A glass column, 1.9 m (6 ft) × 2 mm i.d., was packed with 3% of a 50% phenyl-substituted methyl silicone on silanized, acid-washed, flux calcined diatomite[4].

Typical Conditions—The gases were hydrogen at 1.55 kg/cm^2 (22 psi) and air at 2.11 kg/cm^2 (30 psi) adjusted to obtain maximum response and helium at 3.52 kg/cm^2 (50 psi) with a flow rate of about 30 ml/min. The temperatures were column, 60°; and injector and detector, about 150°. The column temperature and carrier flow rates were adjusted to obtain the first peak preferably within 5 min and complete resolution of the peaks. The current was 2×10^{-11} amp full-scale deflection (fsd) or was adjusted to obtain peak heights greater than 50% fsd, depending on peak sharpness.

Standard Solution—Weigh accurately about 25 mg of *N,N*-dimethylaniline base in a 25-ml volumetric flask. Add 1 ml of concentrated hydrochloric acid and about 10 ml of water. Shake to dissolve into one phase and dilute to volume. Use this solution to make standard solutions of varying concentrations ranging from 1.5 to 1500 μg/ml.

Internal Standard Solution—Dissolve about 5 mg of naphthalene in 100 ml of cyclohexane.

Sample Preparation—Weigh accurately about 1 g of bulk sample into a conical 15-ml centrifuge tube. Add 5 ml of 5% NaOH and stir on a mixer until dissolved. For the recovery study, add 1.00 ml of a standard dimethylaniline solution, mix thoroughly into a suspension, and then add 4 ml of 5% NaOH to dissolve.

Procedure—Add 1.00 ml of the cyclohexane solution, shake vigorously for about 1 min, and allow the phases to separate, centrifuging if necessary. Carefully draw a 1-μl sample from the upper cyclohexane phase and inject it into the chromatograph. For greater accuracy, make comparisons with standards having concentrations of the same order of magnitude

[1] Although this method was developed independently, it is similar in principle to those subsequently submitted in confidence from several manufacturers; it differs in details, however.
[2] Perkin-Elmer model 900.
[3] Infotronics CRS-100.
[4] OV-17 on 100–120-mesh Gas Chrom Q.