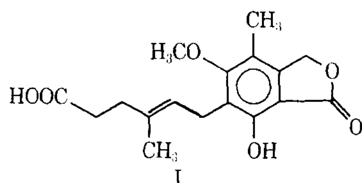
Determination of Mycophenolic Acid and Its Glucuronide Metabolite in Plasma

R. J. BOPP, R. E. SCHIRMER▲, and D. B. MEYERS

Abstract □ GLC and fluorometric procedures were developed for the determination of mycophenolic acid and its principal metabolite, mycophenolic acid glucuronide, in plasma. The GLC procedure consists of extraction of the compound, silylation with trimethylsilylimidazole in carbon disulfide, and chromatography of the sample on a 2% methylphenylsilicone fluid column at 270°. The silyl derivative of lithocholic acid is used as an internal standard. The fluorescence procedure consists of an extraction followed by measurement of the fluorescence of the compound in pH 10.0 buffer, with an excitation wavelength of 350 nm. and an emission wavelength of 438 nm. The accuracy of both procedures is better than 10%.

Keyphrases □ Mycophenolic acid and glucuronide—GLC and fluorometric analyses, human plasma □ GLC—analysis, mycophenolic acid and glucuronide in plasma □ Spectrophotofluorometry—analysis, mycophenolic acid and glucuronide in plasma

Mycophenolic acid (I) is an antibiotic isolated from



penicillium cultures (1, 2) and reported to have antibacterial and antifungal activity (3, 4) as well as antiviral and antitumor activity (5–9). Methods for identifying mycophenolic acid by TLC (10) and for quantitating it by GLC (11) have been reported in the literature. The only methods reported for determining mycophenolic acid in biological samples have been turbidimetric microbiological assays (3, 12–14). There are two major drawbacks to the microbiological assays:

1. They do not allow the relative amounts of mycophenolic acid and its major metabolite, the phenolic glucuronide[1], to be determined.
2. Under actual clinical conditions, a patient typically receives several drugs in addition to the one being studied, and the effects of these other drugs and their metabolites on the precision and accuracy of a microbiological assay are not easily assessed, nor can the assay result be corrected for such effects if they are found to be significant.

For these reasons, more direct analytical procedures have been developed for mycophenolic acid and its glucuronide in biological samples.

This paper reports both a fluorometric and a GLC procedure for the determination of mycophenolic acid in human plasma. Both procedures have been used to analyze for mycophenolic acid in other types of biological samples including spinal fluid, urine, tissue, and tissue culture media; other substances present in these samples do not interfere with the assay. The fluorometric method has the advantage of being faster and is, therefore, better suited to handling large numbers of samples. The GLC method, on the other hand, had the advantage of being less susceptible to interference from other compounds.

EXPERIMENTAL

GLC Procedure—*Reagents*—The following were used:

1. Trimethylsilylimidazole—50 μl. of trimethylsilylimidazole[2] dissolved in 1 ml. of carbon disulfide (analytical reagent). This solution must be prepared fresh daily and should be kept protected from moisture and air.
2. β-Glucuronidase—used as supplied[3].
3. Lithocholic acid standard solution—30 mcg./ml. in dichloroethane (analytical reagent).
4. Sodium mycophenolate standard solution—20 mcg./ml. in distilled water.
5. Acetate buffer, pH 5.0—0.2 *M*.
6. Borate buffer, pH 10.0—0.5 *M*.

Conditions—A gas chromatograph[4] equipped with a flame-ionization detector was used for the analysis. The column was a 1.2-m. (4-ft.) glass U-tube (3-mm. i.d.) packed with 2% methylphenylsilicone fluid (OV-17) on 80–100-mesh diatomite (high performance Chromosorb G) and was operated isothermally at 270° with helium for the carrier gas. The flash heater was operated at 310° and the flame detector was operated at 300°. The retention time of mycophenolic acid silyl derivative was 4.8 min., and the retention time of the lithocholic acid silyl derivative was 8.2 min. under these conditions. A typical chromatogram is presented in Fig. 1.

Standard Preparation—Place 0.0, 0.10, 0.30, 0.60, 1.00, and 1.50 ml. of sodium mycophenolate standard solution in six 15-ml. screw-cap centrifuge tubes. In the assay for total drug, 0.1 ml. of blank plasma and 0.9 ml. of normal saline are added to each tube; for free drug, 1.0 ml. of blank plasma is added to each.

Sample Preparation—A 1.0-ml. sample of undiluted plasma is used in the analysis for free mycophenolic acid, while a 1.0-ml. aliquot of plasma that has been diluted 1:10 with normal saline is used in the analysis for total mycophenolic acid.

All glassware used in handling the standards and samples should be cleaned with chromate cleaning solution and siliconized with a 1% solution of dimethyldichlorosilane in toluene.

Procedure for Total Mycophenolic Acid—The samples and standards are adjusted to pH 5.0 with acetate buffer, and 5220 units of β-glucuronidase are added to each[3]. The samples are incubated at 37° for at least 1 hr. and may be incubated overnight if convenient. More than 90% of the glucuronide is hydrolyzed within 10 min. under these conditions.

Add 1.0 ml. of lithocholic acid standard solution and 9.0 ml. of dichloroethane to each tube, shake for 15 min., and centrifuge to separate the phases. Discard the aqueous layer and transfer the dichloroethane layer to a 20-ml. centrifuge tube. Add 8 ml. of 0.1 *M* sodium hydroxide to each tube, and shake and centrifuge as before. Transfer the aqueous layer to another 20-ml. centrifuge tube, add

[1] The metabolism of mycophenolic acid has been studied by M. J. Sweeney, D. H. Hoffman, and M. A. Esterman of the Lilly Research Laboratories.

[2] Pierce Chemical Co.

[3] Glusulase (Endo Laboratories, 174,000 units glucuronidase/ml.). Ketodase (5000 units glucuronidase/ml.) can be used in place of Glusulase in the fluorometric assay, but it contains an impurity which interferes in the GC analysis.

[4] F & M model 402.

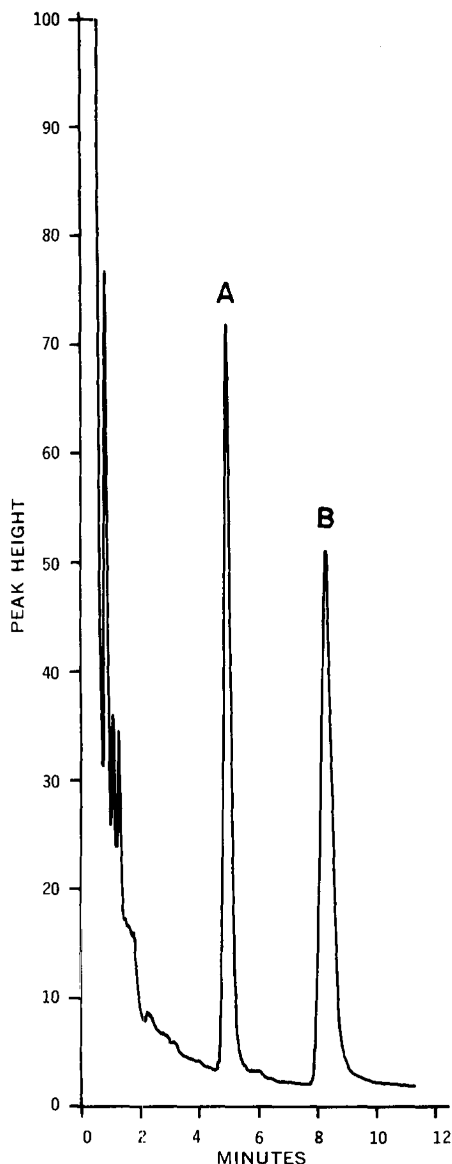


Figure 1—*Gas-liquid chromatogram of trimethylsilylated mycophenolic acid (A) and trimethylsilylated lithocholic acid (B). See text for conditions.*

1.0 ml. of 1 *M* hydrochloric acid, mix, and let stand for 10 min. Extract with 10 ml. of spectroquality chloroform, transfer the chloroform layer to a 15-ml. screw-cap centrifuge tube, and evaporate to dryness. Rinse the sides of the tube once with 0.5 ml. of chloroform and evaporate to dryness again. Add 40 μl. of trimethylsilylimidazole-carbon disulfide reagent to each sample, mix, and inject approximately 3 μl. onto the GLC column.

Procedure for Free Mycophenolic Acid—The procedure for free mycophenolic acid is the same as for total mycophenolic acid except that the incubation with β-glucuronidase is deleted.

Precision and Accuracy—The precision and accuracy of the GLC procedure were determined by analyzing plasma samples spiked with known amounts of mycophenolic acid. The results are summarized in Table I.

Spectrophotofluorometric Procedure[5]—*Reagents*—The following were used:

1. 0.05 *M* $FeCl_3$ solution—8.1 g. of ferric chloride dissolved in 1 l. of distilled water. The pH of this solution should be 2.0 or lower and may be adjusted with hydrochloric acid if necessary.

[5] All fluorometric measurements were made on an Aminco-Bowman spectrophotofluorometer equipped with a Hanovia 901C-11 high pressure xenon lamp and an R-136 photomultiplier tube.

Table I—Precision and Accuracy of the GLC Procedure for Free Mycophenolic Acid

Nominal Concentration, mcg./ml.	Number of Samples Analyzed	Relative Standard Deviation, %	Relative Error, %
5	14	2	−4
10	5	5	+3
15	5	10	+10

2. β-Glucoronidase, pH 5.0 acetate buffer, and pH 10.0 borate buffer—used or prepared as described in the GLC procedure.

3. Sodium mycophenolate standard solutions—standard solutions of 10 and 40 mcg./ml. nominal concentration prepared in distilled water.

Standard Preparation—The standards for the total mycophenolic acid assay are prepared by pipeting 1.0 ml. of blank plasma into each of six 50-ml. centrifuge tubes and pipeting 1.0, 0.75, 0.50, 0.25, and 0.125 ml. of the 40-mcg./ml. standard solution into tubes 1 through 5, respectively. The sample in tube 6 serves as the blank. The standards for free mycophenolic acid assay are prepared in the same manner except that the 10-mcg./ml. sodium mycophenolate standard solution is substituted for the 40-mcg./ml. solution.

Samples—One (1.0) milliliter of plasma is required to assay for free mycophenolic acid, and another 1.0-ml. sample is required to analyze for total mycophenolic acid.

Procedure for Total Mycophenolic Acid—Add 2.0 ml. of pH 5.0 acetate buffer and 5220 units β-glucuronidase to each sample and standard. Incubate for at least 1 hr. at 37° and extract with 10 ml. of dichloroethane. Discard the aqueous phase and wash the dichloroethane with 2.0 ml. of ferric chloride reagent. Discard the aqueous layer and transfer 5.0 ml. of the dichloroethane solution to a 20-ml. screw-cap centrifuge tube containing 10 ml. of the pH 10 borate buffer. Extract and measure the fluorescence of the aqueous phase with the excitation wavelength set at 350 nm. and the emission wavelength set at 438 nm. A typical fluorescence spectrum is shown in Fig. 2.

Procedure for Free Mycophenolic Acid—The procedure for free mycophenolic acid is the same as for total mycophenolic acid except that the addition of β-glucuronidase and the incubation at 37° are deleted.

Precision and Accuracy—The precision and accuracy of the procedure were determined in two concentration ranges. In the low range, 10 spiked plasma samples containing from 0.41 to 4.10

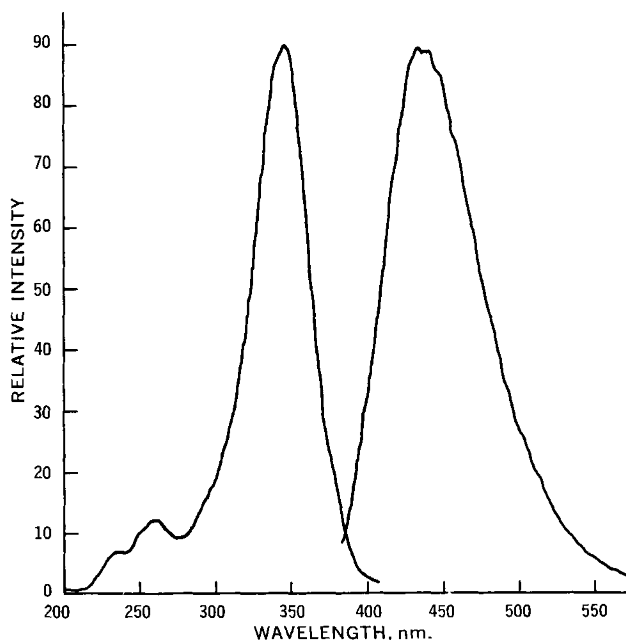


Figure 2—*Fluorescence spectrum of an 8-mcg./ml. solution of mycophenolic acid in pH 10 borate buffer USP.*

Table II—Hydrolysis of Mycophenolic Acid Glucuronide

	Percent Glucuronide Hydrolyzed after:					
pH	1 hr. with Glucuronidase	2 hr. without Enzyme	4 hr. without Enzyme	6 hr. without Enzyme	18 hr. without Enzyme	24 hr. without Enzyme
1.2	0.9	1.8	0.6	0.9	0.7	0.6
5.0	97.8	0.7	0.4	0.4	0.4	0.3
6.0	103.2	0.7	0.4	0.4	0.4	0.5
7.0	101.3	0.6	0.5	0.4	0.4	0.4
8.0	101.3	2.9	0.4	0.4	0.4	0.4
10.0	1.4	0.6	0.5	0.4	0.4	0.4

Table III—Efficiency of the Extractions

Extract from	Extract into	Percent Recovery
2.0 ml. of:		
pH 4.0	10 ml. of dichloroethane	100.0
pH 5.0		98.0
pH 6.0		94.5
pH 7.0		90.0
5.0 ml. of dichloroethane	10 ml. of:	
	pH 7.0	23.4
	pH 8.0	58.2
	pH 9.0	88.7
	pH 10.0	91.6

mcg./ml. mycophenolic acid were analyzed using a calibration curve covering the concentration range 0.0–1.0 mcg./ml. in 0.2-mcg./ml. increments. In the high concentration range, 10 spiked plasma samples in the 4.10–103.0-mcg./ml. range were analyzed using a calibration curve as described for total mycophenolic acid assay. If the fluorescence intensity of a sample was off-scale, a dilution with pH 10.0 buffer was made immediately and the intensity was measured again, as is done with clinical samples when necessary. Sample concentrations outside the calibration curve were intentionally used in this study in order to include the dilution errors that would be present in clinical samples in these estimates of the precision and accuracy. The lower concentration range gave a relative standard deviation of ±20% with a relative error of −3.1%, while the higher concentration range gave a relative standard deviation of ±18% and a relative error of +9%.

Hydrolysis of Mycophenolic Acid Glucuronide—A preliminary study of enzymatic hydrolysis of the phenolic glucuronide of mycophenolic acid was performed by carrying aliquots of a plasma sample through the fluorometric procedure but varying the time of incubation with glucuronidase from 10 min. to overnight (16 hr.). Approximately 91% of the glucuronide was hydrolyzed within 10 min. and 100% within 30 min. A 1-hr. incubation with glucuronidase was selected for routine use to ensure complete hydrolysis in all cases. This study also demonstrated that the incubation could be carried out overnight if necessary without affecting the accuracy of the assay.

A second study was carried out to determine the effect of pH on the hydrolysis of the glucuronide both in the presence and absence of glucuronidase. Solutions containing 40.0 mcg./ml. of mycophenolic acid glucuronide were incubated at several pH's and analyzed at several different times using the fluorometric procedure. The results are presented in Table II. No significant hydrolysis occurred in the absence of enzyme over the 1.0–10.0 pH range and over periods of at least 24 hr. In the presence of enzyme, the hydrolysis was complete within 1 hr. at pH's between 5.0 and 8.0, but the rate of hydrolysis decreased very rapidly outside of this range.

Extraction Efficiency—Standard solutions of mycophenolic acid were prepared in dichloroethane and in 0.2 *M* acetate and phosphate buffers at pH's of 4.0, 5.0, 6.0, and 7.0. Samples (2.0-ml.) of the aqueous standards were extracted with 10 ml. of dichloroethane, and the amount of drug extracted was determined by measuring the UV absorbance of the dichloroethane fractions and standard solution at 303 nm. The results are recorded in Table III.

Aliquots (5.0 ml.) of the standard solution in dichloroethane were then extracted with 10.0-ml. aliquots of 0.2 *M* phosphate buffer at pH 7.0 or 8.0 or with 10.0-ml. aliquots of 0.5 *M* borate buffer at pH 9.0 or 10.0. A 5.0-ml. aliquot of the aqueous phase was transferred by pipet to a 10.0-ml. volumetric flask and diluted to volume with 0.1 *M* sodium hydroxide. The amount of mycophenolic acid extracted was then determined by measuring the UV absorbance of the diluted samples at 342 nm. against standards prepared in 0.1 *M* sodium hydroxide. The results of these studies are also included in Table III.

DISCUSSION

Both free mycophenolic acid and the total of free mycophenolic acid and mycophenolic acid glucuronide are determined by the procedures presented in this paper. Free mycophenolic acid is initially separated from the glucuronide in the dichloroethane extraction. Additional specificity is obtained because the glucuronide does not chromatograph the same as the free acid and is only about 3% as fluorescent as the mycophenolate anion. There is no measurable hydrolysis of the glucuronide to the free acid under any of the conditions used in this assay except in the presence of glucuronidase.

The glucuronide is the only significant metabolite of mycophenolic acid in man, so total mycophenolic acid is determined by cleaving the glucuronide enzymatically and then following the procedure for free mycophenolic acid. The enzymatic cleavage has been shown to be rapid and complete.

The fluorometric procedure is not as specific as the GLC procedure, and some caution must be exercised in using it. In particular, other fluorescent phenolic compounds are potential interferences. The ferric chloride backwash was introduced into the assay procedure to remove salicylates which were found to be common interferences in clinical samples. While mycophenolic acid has been reported to form a complex with the ferric ion (15, 16), there is no loss of the compound in the backwash step, as was determined by carrying standard samples through the procedure with and without the backwash step and comparing the results. The presence of interfering compounds can usually be detected by changes in the position or shape of the fluorescence excitation and emission spectra.

Another disadvantage of the fluorescence procedure is that the fluorescence intensity must be measured within 30 min. of extracting the drug into the pH 10 buffer. Losses in fluorescence intensity of 10% or more may occur after that time. These losses may be associated with partial opening of the lactone ring (15, 16), but this point has not been investigated.

Analysis of samples by both the fluorescence and GLC procedures has been shown to give the same results within experimental error; this is expected as the relative errors already presented are very similar for the two methods. The trend seen in the relative error as a function of sample concentration is probably not due to any systematic error in the detection step of the assay, because systematic errors would not be the same for methods as different as flame-ionization and fluorescence detection. The nonlinear calibration can probably be attributed to incomplete extractions, so introducing multiple extractions at the appropriate steps might improve the accuracy of the analysis if desired. Finally, it should be pointed out that the large relative standard deviation obtained for the fluorometric procedure contains a significant contribution from variations in the intensity of light from the high pressure xenon lamp. The stability of this light source decreases with the age of the lamp, so the relative standard deviation could be improved either by using a new lamp or by replacing the xenon lamp with another, more stable light source. With a stable light source, the relative standard deviation would probably be closer to 10% than to the value reported here.

REFERENCES

(1) B. Gosio, *Rig. Ig. Sanita Pubb. Ann.*, **7**, 825(1896).

(2) C. L. Alsberg and O. F. Black, *U. S. Dept. Agr. Bur. Plant Ind. Bull.*, **270**, 7(1913).

(3) H. W. Florey, K. Gilliver, M. A. Jennings, and A. G. Sanders, *Lancet*, **1**, 46(1946).

(4) K. Gilliver, *Ann. Bot. (London)*, **10**, 271(1946).

(5) K. Ando, S. Suzuki, G. Tamura, and K. Arima, *J. Antibiot. (Tokyo)*, **21**, 649(1968).

(6) R. H. Williams, D. H. Lively, D. C. DeLong, J. C. Cline,

M. J. Sweeney, G. A. Poore, and S. H. Larsen, *ibid.*, **21,** 463(1968).
(7) S. B. Carter, T. J. Franklin, D. F. Jones, B. J. Leonard, S. D. Mills, R. W. Turner, and W. B. Turner, *Nature*, **223,** 848(1969).
(8) D. N. Planterose, *J. Gen. Virol.*, **4,** 629(1969).
(9) R. H. Williams, L. D. Boeck, J. C. Cline, D. C. DeLong, K. Gerzon, R. S. Gordee, M. Gorman, R. E. Holmes, S. H. Larsen, D. H. Lively, T. R. Mathews, J. D. Nelson, G. A. Poore, W. M. Stark, and M. J. Sweeney, *Antimicrob. Ag. Chemother.*, **1968,** 229.
(10) F. E. Gainer and R. L. Hussey, *J. Chromatogr.*, **54,** 446 (1971).
(11) F. E. Gainer and H. J. Wesselman, *J. Pharm. Sci.*, **59,** 1157 (1970).
(12) T. Noto, M. Sawada, K. Ando, and K. Koyama, *J. Antibiot.*, **22,** 165(1969).
(13) F. Kavanaugh, *Advan. Appl. Microbiol.*, **2,** 65(1960).
(14) T. Noto, Y. Harada, and K. Koyama, *J. Antibiot. (Tokyo)*, **23,** 96(1970).
(15) P. W. Clutterbuck and H. Raistrick, *Biochem. J.*, **27,** 654 (1933).
(16) J. H. Birkinshaw, A. Bracken, E. N. Morgan, and H. Raistrick, *ibid.*, **43,** 216(1948).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 23, 1972, from *Eli Lilly and Company, Indianapolis, IN 46206*
Accepted for publication June 7, 1972.
▲ To whom inquiries should be directed.

Chlorpromazine Metabolism V: Disposition of Free and Conjugated Metabolites in Blood Fractions of Schizophrenic Patients

PUSHKAR N. KAUL▲, MAHARAJ K. TICKU, and MERVIN L. CLARK

Abstract ☐ Disposition of the circulating levels of 10 chlorpromazine metabolites and their conjugates in the blood fractions of chronic schizophrenic patients was determined. The erythrocytes were found to sequester nearly 50% of the total blood concentration of the free metabolites, whereas the platelets contained only insignificant amounts. Of particular significance was the localization of the conjugated metabolites in the red cells. The implications of sequestration of the metabolites and their conjugates by the erythrocytes are discussed.

Keyphrases ☐ Chlorpromazine metabolism—disposition of free and conjugated metabolites in blood fractions of schizophrenic patients ☐ Erythrocyte sequestration—disposition of free and conjugated metabolites of chlorpromazine in blood of schizophrenic patients ☐ Blood distribution, erythrocyte sequestration—chlorpromazine metabolites, schizophrenic patients ☐ Schizophrenics' chlorpromazine blood distribution—disposition of free and conjugated metabolites, erythrocyte sequestration

Although metabolism of chlorpromazine has been studied extensively in the past decade (1–5), very little is known about the disposition of the drug and/or its metabolites in the blood. Only recently has it been possible to determine the blood levels of a majority of the metabolites (6). Earlier failures in consistently quantitating the circulating levels of the metabolites have been, at least in part, due to nonavailability of sensitive and precise assay methodology. Another possible reason for such failures might have been that investigators used plasma or serum samples for measuring the blood levels, which would tend to be low if the blood cells sequestered the drug and its metabolites.

For clinical studies correlating therapeutically available levels of a drug (and/or its metabolites) with its administered dose, it is important to consider the total concentration in the whole blood rather than only in the plasma. Both the protein-bound and the free (nonprotein-bound) drug molecules present in the cells as well as the plasma usually exist in a steady-state equilibrium of the type proposed in Fig. 1. The extent of delivery of the therapeutically active molecules (drug and/or its metabolites) at the biophase is dependent on the free plasma levels. The duration of this delivery, however, should be dependent not only on the free plasma levels but also on the bound plasma levels as well as on the free and bound concentrations localized in the blood cells. Therefore, to study the contribution of the blood cells in monitoring the therapeutic availability of a drug and/or its metabolites, a detailed disposition of these molecules in the blood fractions is necessary.

Huang and Ruskin (2) could not detect any chlorpromazine-like material in erythrocytes of patients receiving chlorpromazine. Hammar and Holmsted (3) also failed to detect any nonconjugated metabolites in the erythrocytes. Zingales (7), however, was the first to detect qualitatively the conjugated nonphenolic metabolites of chlorpromazine in the plasma and in erythrocyte washings and hemolysates, but to date no quantitative data have been reported.

In previous studies dealing with the development of the dansylation assay (8) and its application to blood (6),