GLC of Mycophenolic Acid and Related Compounds

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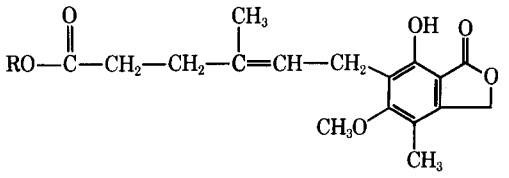
Abstract □ A GLC method is described for the quantitative determination of mycophenolic acid and monosodium mycophenolate. Data are presented to show that both the methyl ester and ethyl ester derivatives of mycophenolic acid can be chromatographed along with a cyclic acid hydrolysis product of mycophenolic acid. In addition, quantitative results are presented for the assay of mycophenolic acid and the monosodium salt in various formulations. Spectral and TLC results are also included.

Keyphrases □ Mycophenolic acid, related compounds—determination □ TLC—separation □ GLC—analysis □ Tetraphenylethylene, chloroform dissolved—internal standard □ IR spectrophotometry—structure □ NMR spectroscopy—structure

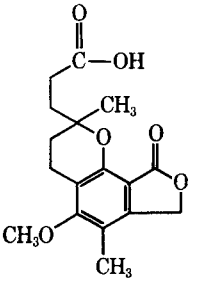
Mycophenolic acid (Structure I) is an antibiotic fermentation product which is isolated from penicillium cultures (1, 2). The antibiotic is known to have



I, R = H
II, R = Na
III, R = CH_3
IV, R = C_2H_5

antibacterial and antifungal properties which have been studied previously (3, 4). Recently, two groups of researchers (5, 6) reported on the antitumor activity of mycophenolic acid, while Williams *et al.* (7) reported that the substance exhibited both antiviral and antitumor properties. Work has been done, both chemical and physical in nature, to elucidate the structure of mycophenolic acid (8, 9) and some related compounds, which were isolated from the culture filtrates of a strain that produces mycophenolic acid (10). Birch and Wright (11) have devised a scheme for the total synthesis of mycophenolic acid. This paper reports the quantitative determination of mycophenolic acid and related compounds by GLC.

Ethyl mycophenolate (IV) may be formed during the isolation of mycophenolic acid from penicillium cultures (12) and recrystallization of the acid by interaction of the acid and ethanol. However, Campbell *et al.* (10) have reported the isolation of ethyl mycophenolate from the culture filtrates of a strain that produces mycophenolic acid. The cyclic compound, 3,4-dihydro-5 - methoxy - 2,6 - dimethyl - 9(7*H*) - oxo - 2*H* - furo-⟨3,4-*h*⟩benzopyran-2-propionic acid (V) was isolated from the reaction vessel after heating the acid at 100° in 10% hydrochloric acid for 18 hr. Methanol leads to partial esterification of mycophenolic



V

acid during extraction processes (12), probably resulting in methyl mycophenolate (III). Monosodium mycophenolate (II) may be formed by adjusting a slurry of mycophenolic acid to pH 7–8 with sodium hydroxide.

No record was found in the literature, either directly or indirectly, reporting the quantitative determination of mycophenolic acid.

EXPERIMENTAL

Equipment—A gas chromatograph (Hewlett-Packard model 402) equipped with a flame-ionization detector was used for the experimental work. The detector signal was fed to a 1-mv. recorder (Honeywell Electronik 16) with a chart speed of 15 in./hr. and a 1-sec. full-scale response. Samples were injected with a 10-μl. syringe (Hamilton No. 701).

Materials—Helium was used as a carrier gas, while electrolytic hydrogen and oxygen were used in the detector. The stationary phase was 3.8% Linde W-98 silicone gum applied by the solution technique to silanized diatomaceous earth (Diatoport S) (80/100 mesh) and packed in a borosilicate glass column, 0.91 m. × 0.64 cm. o.d. Chloroform (Reagent ACS) was used to dissolve the tetraphenylethylene (Reagent ACS) internal standard. The silylating reagent was bis(trimethylsilyl)trifluoroacetamide.[1] Concentrated hydrochloric acid (Reagent ACS) and absolute methanol were employed.

Operating Conditions—The column was operated isothermally at 235°, with the detector block at 280° and the sample injection port at 280°. The helium flow rate was 55 ml./min., with an inlet pressure of 40 psig. Oxygen and hydrogen flow rates were 200 and 50 ml./min., respectively. The electrometer range was 10, with an attenuation of 128. Sample injections of 1.5 μl. were used throughout the study.

Quantitative Analysis—Tetraphenylethylene, 25 mg. in 100 ml. of chloroform, is used as an internal standard.

Mycophenolic Acid—Accurately weigh 50 mg. of mycophenolic acid into a 50-ml. volumetric flask, and dilute to volume with chloroform. Pipet 1.0 ml. of the solution into a 3-ml. butyl-rubber-stoppered ampul, and add 1.0 ml. of the internal standard solution. Carefully evaporate the solution in the ampul to dryness, using a gentle stream of nitrogen and a warm water bath. Add 0.5 ml. of bis(trimethylsilyl)trifluoroacetamide to the ampul, and seal before heating for 1 hr. in a heating block at 80°. Cool to room temperature before chromatographing. Prepare a standard reference solution by accurately weighing 50 mg. of mycophenolic acid reference standard into a 50-ml. volumetric flask and diluting to volume with chloroform. Treat this solution exactly as the sample solution.

[1] Regis, Regisil Chemical Co., Chicago, Ill.

Table I—Mycophenolic Acid Assay Results

Sample	Theory	Found	n	$RSD \pm \%$	$RE\ \%$
Raw material	1000 mg./g.	997.5 mg./g.	12	1.81	−0.25
Raw material	1000	999.3	3		−0.07
Raw material	1000	1002.0	3		+0.20
Capsules with starch	200 mg./capsule	204.4 mg./capsule	5	4.08	+2.18
Capsules with starch	200	198.7	5	1.12	−0.65
Mycophenolic acid + microcrystalline cellulose	570 mg./g.	569.5 mg./g.	3		−0.09
Mycophenolic acid + talc	470	469.3	3		−0.15

Table II—Monosodium Mycophenolate Assay Results

Sample	Theory	Found	n	$RSD \pm \%$	$RE\ \%$
Ampul (10% aqueous)	100 mg./ml.[a]	102.6 mg./ml.[a]	5	1.71	+2.60
Ampul (10% aqueous)	100	103.1	9	2.30	+3.10
Capsules with starch	200 mg./capsule	197.4 mg./capsule	5	4.40	+1.80
Capsules with starch	200	201.0	5	0.69	+0.50
Monosodium mycophenolate + talc (1:1)	500 mg./g.	509.8 mg./g.	3		+1.96

[a] Mycophenolic acid equivalency per unit sample.

Mycophenolic Acid Capsules, 200 mg.—Weigh five filled capsules and empty the contents into a small beaker. Wash the empty capsules with chloroform, discarding the washings. Allow the capsules to dry before weighing. Subtract the weight of the empty capsules from that of the filled capsules to determine the average fill weight. Thoroughly mix the dry powder in the beaker, and accurately weigh a portion of the sample equivalent to 100 mg. of mycophenolic acid. Quantitatively transfer the sample to a 125-ml. separator containing 20 ml. of purified water. Extract the compound with three 20-ml. portions of chloroform, allowing 2 min. for each extraction. Filter the chloroform layer through anhydrous sodium sulfate into a 100-ml. volumetric flask. Wash the sodium sulfate with chloroform, and collect the washings in the flask. Dilute to volume with chloroform and shake well. Pipet 1.0 ml. of this solution into a 3-ml. butyl-rubber-stoppered ampul, and proceed as directed for mycophenolic acid, using the same internal standard and standard reference solution.

Monosodium Mycophenolate—Accurately weigh 100 mg. of sodium mycophenolate into a 100-ml. volumetric flask containing 50 ml. of methanol. Add 0.5 ml. of concentrated hydrochloric acid to the flask and shake well. Dilute to volume with methanol and shake well before transferring 10 ml. of the solution to a butyl-rubber-stoppered ampul. Seal the ampul and place it in a heating block at 80° for 0.5 hr. Cool the contents of the ampul to room temperature. Pipet 1.0 ml. of the solution into a 3-ml. butyl-rubber-stoppered ampul and proceed as with mycophenolic acid. Prepare a standard reference solution by accurately weighing 100 mg. of monosodium mycophenolate standard into a 100-ml. volumetric flask containing 50 ml. of methanol. Treat this solution exactly as the sample solution.

Monosodium Mycophenolate Ampuls, 10%—Pipet 1.0 ml. of the ampul solution into a 100-ml. volumetric flask containing 50 ml. of methanol. Proceed as directed for monosodium mycophenolate, using the same internal standard solution and standard reference solution.

Experimental procedures and details for the GLC of ethyl mycophenolate, methyl mycophenolate, and the cyclic compound are the same as for mycophenolic acid.

Chromatograph the standard and sample solutions, and measure the peak heights of the respective internal standard, standard, and sample peaks.

Calculations—

$$\frac{\text{peak height sample}}{\text{peak height sample internal standard}} = R_1 \qquad \text{(Eq. 1)}$$

$$\frac{\text{peak height standard}}{\text{peak height standard internal standard}} = R_2 \qquad \text{(Eq. 2)}$$

$$\frac{R_1 \times \text{milligram standard}}{R_2 \times \text{milligram sample}} \times \text{standard purity milligram/gram} = \text{milligram active ingredient/gram sample} \qquad \text{(Eq. 3)}$$

Substitute milliliter sample for milligram sample when solutions are assayed.

$$\text{Eq. 3} \times \text{average fill weight of capsule} = \text{milligram active ingredient/capsule} \qquad \text{(Eq. 4)}$$

If the active ingredient is monosodium mycophenolate, the acid equivalency can be obtained by multiplying Eqs. 3 and 4 by the factor 0.935.

RESULTS AND DISCUSSION

Each of the compounds studied, except monosodium mycophenolate, is chloroform soluble; thus, it was convenient to do simple extractions and dilutions with this solvent. Monosodium mycophenolate is very soluble in water and relatively insoluble in most organic solvents except methanol, which was used for this compound.

Since it is more difficult to silylate the sodium salt of a carboxylic acid than the acid itself, initial efforts were made to convert monosodium mycophenolate in methanolic solution to mycophenolic

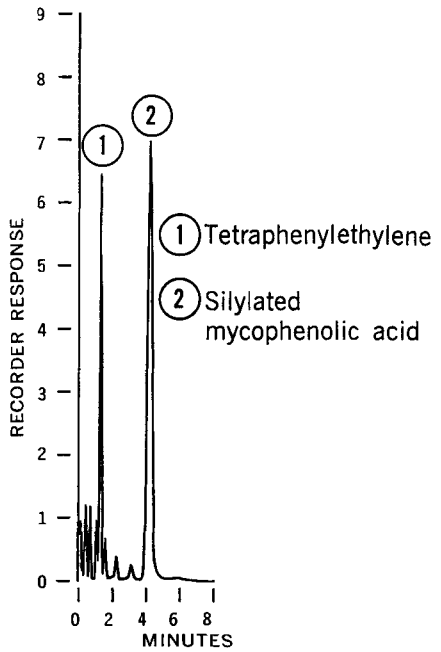


Figure 1—*Typical chromatogram of internal standard and sample.*

Table III—Retention Times of Trimethylsilyl Ethers of Mycophenolic Acid and Related Compounds

Compound	Retention Time,[a] min.
Mycophenolic acid	4.0
Monosodium mycophenolate (as methyl mycophenolate)	3.2
Cyclic compound	4.6
Ethyl mycophenolate	3.6
Methyl mycophenolate	3.2
Tetraphenylethylene[b]	1.2

[a] Using chromatographic parameters described under operating conditions. [b] Internal standard.

acid by the addition of concentrated hydrochloric acid with shaking. This resulted in a solution containing two compounds, as shown by GLC and TLC. The chromatograms suggested that the compounds were methyl mycophenolate and mycophenolic acid. Both IR and NMR confirmed that one component was methyl mycophenolate. Additional checks were not made to determine whether the second component was mycophenolic acid; but the chemistry of the system, along with stability observations, indicated that it was the acid. Since methanol and hydrochloric acid are conducive to ester formation, it was convenient to form methyl mycophenolate from monosodium mycophenolate instead of forming the acid. TLC and GLC of aliquots taken from the reaction mixture at various time intervals indicated that esterification was taking place at a measurable rate even at room temperature. It was possible to obtain complete esterification by heating the reaction mixture at 80° for 0.5 hr. Again, TLC and GLC confirmed that the solution contained only one component, methyl mycophenolate. Chromatography also indicated that no formation and subsequent esterification of the cyclic acid hydrolysis compound (V) were occurring. The methylated cyclic compound has a lower R_f value and a longer retention time than methyl mycophenolate.

To confirm that the sodium salt was being converted to the ester, an alternate method of preparation was employed in which a methanolic solution of mycophenolic acid was treated with gaseous hydrogen chloride. IR and NMR confirmed that the product was methyl mycophenolate. The product, from both methods of preparation, had the same R_f value by TLC.

It was found necessary to heat all compounds studied during the silylating reaction to obtain consistent results. Assay results for mycophenolic acid and monosodium mycophenolate were reproducible, and recoveries were good. Table I shows the results of assays of several lots of mycophenolic acid raw material and formulations. Assay results for monosodium mycophenolate are shown in Table II.

The response of the detector is linear over a range of concentrations from 0.5 to 1.5 mg./ml. for the silylated derivatives of mycophenolic acid and methyl mycophenolate. The continual use of the silylating agent causes a deposit to form on the detector anode, which eventually causes a loss in precision and accuracy. This problem can be eliminated by periodically cleaning the anode in an ultrasonic bath.

Both mycophenolic acid and monosodium mycophenolate are very stable compounds which lend themselves to quantitative assays as silylated derivatives. A typical chromatogram, showing peaks for the internal standard and silylated mycophenolic acid, is shown in Fig. 1. Although no quantitative work was done on ethyl mycophenolate and the cyclic compound, it should be possible to quantitate these compounds by using the same experimental parameters used for mycophenolic acid. Metabolites, other than ethyl mycophenolate, that are related to mycophenolic acid (10) also should be amenable to GLC. It is significant that mycophenolic acid, related metabolites, and other related compounds can be chromatographed by using very similar experimental conditions. Table III presents the retention times for the compounds studied.

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