Decomposition of Thimerosal in Aqueous Solution and its Determination by High-Performance Liquid Chromatography

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Abstract □ Studies on the decomposition of thimerosal in aqueous solution have confirmed that thiosalicylic acid and ethylmercuric hydroxide are the initial products. On prolonged reaction, thiosalicylic acid was oxidized to 2,2′-dithiosalicylic acid, while ethylmercuric hydroxide was reduced to elemental mercury. As a result, a specific, reverse-phase high-performance liquid chromatographic assay has been developed for thimerosal in the presence of its decomposition products. By comparison, an existing colorimetric assay procedure employing dithizone was shown to be not fully specific. The presence of sodium chloride in the solution accelerated the decomposition of thimerosal. There was evidence that thimerosal was sorbed onto plastic containers on storage.

Keyphrases □ Thimerosal—degradation products, aqueous solutions, effect of sodium chloride, high-performance liquid chromatography □ Ophthalmic solutions—thimerosal decomposition in aqueous solutions, effect of sodium chloride, high-performance liquid chromatography □ High-performance liquid chromatography—thimerosal and its degradation products, aqueous solutions, effect of sodium chloride

Thimerosal, ethyl (sodium *o*-mercaptobenzoato)mercury (I), is widely used in pharmaceutical preparations, such as eyedrops, as an antibacterial and antifungal preservative. However, it is known to be unstable in aqueous solution (1, 2). It has also been reported that the presence of halides can have an adverse influence on the stability of I (3–5), and that I can be lost from solutions stored in plastic containers (6). The object of this study was to identify the decomposition products of thimerosal in aqueous solution under various conditions and, hence, develop a specific analytical method for the intact preservative in stored test solutions.

The decomposition of I in aqueous solution has been studied previously (2), and it was shown that the major products were thiosalicylic acid (II) and ethylmercuric hydroxide (III). Compound II can also undergo irreversible oxidation to 2,2′-dithiosalicylic acid (IV) (7). Hydrolysis in the presence of halide ions gave ethylmercuric halide (V) rather than the hydroxide (4).

Chemical assay procedures previously reported for the analysis of I have included UV spectrophotometry (8), polarography (9, 10), and colorimetry (11–13). None of these methods were fully specific for I in the presence of decomposition products, particularly the colorimetric assay, which is sensitive to all mercury-containing species present in the solution. Therefore, it was decided to seek a high-performance liquid chromatographic (HPLC) method for the analysis of I. A previously reported anion exchange HPLC method (14) was considered to be unsuitable for the assay of I in pharmaceutical preparations due to poor peak shape, while another method (15) failed to provide adequate resolution of I from its decomposition products. A more recent method (16) using radial-compression HPLC gave imprecise assays and did not satisfactorily resolve the degradation products of I from each other. The HPLC method described here gave resolution of I from its major decomposition products and has been used to assay I in test solutions and pharmaceutical vehicles.

EXPERIMENTAL

Compounds and Reagents—The following compounds were obtained commercially and used without further purification: thimerosal[1], ethylmercuric chloride[2], thiosalicylic acid[3], and 2,2′-dithiosalicylic acid[4]. All other reagents and solvents were analytical reagent grade[1]. Ethylmercuric hydroxide was synthesized by a published method (17).

High-Performance Liquid Chromatography—*Equipment*—A constant-flow pump[5] was used to deliver the eluant to a stainless steel column packed with 10-μm silica particles bonded with octadecylsilane[6]. Injections were made with a rotary valve injector[7] equipped with a 25-μl loop. No attempt was made to control the column temperature. A variable-wavelength detector[8] set at 222 nm was employed at an attenuation of 0.5 AUFS for 0.01% (w/v) solutions of thimerosal and at 0.05 AUFS for 0.001% (w/v) solutions.

Chromatographic Conditions—The mobile phase consisted of a mixture of methanol–water–phosphoric acid in a ratio of 60:50:1.0. The pressure at a flow rate of 2.6 ml min^{-1} was 1800 psig. Separations were effected isocratically at ambient temperature, and quantification was carried out by comparison of the areas of peaks obtained from test solutions with the area of the peak obtained from a standard thimerosal solution (Fig. 1).

Thin-Layer Chromatography—TLC was carried out on prepared silica gel plates[9] (20 cm × 20 cm × 0.25 mm) with mixtures of chloroform–methanol (1:1) (system A) or butanone–ethanol–acetic acid (70:30:0.1) (system B) as the developing solvents; loadings of thimerosal were usually 50 or 100 μg. Components were detected by viewing under UV light at 254 nm and visualized by spraying with a 0.05% (w/v) solution of diphenylthiocarbazone (dithizone) in chloroform.

Dithizone Colorimetric Assay—A volume of the test solution equivalent to 0.1 mg of I was transferred by pipet to a separator containing 0.5 *M* sulfuric acid (20 ml) and glacial acetic acid (20 ml). Ten percent sodium hydroxide (30 ml) was added followed by toluene (15 ml) and dithizone solution (1 ml). The mixture was shaken and allowed to settle. Most of the aqueous (lower) phase was removed and discarded. Anhydrous sodium sulfate (1 g) was added to the remaining emulsion, which was then filtered through a phase separating paper[10]. The color of the clear solution was measured in a 1-cm cell at 475 nm against a reagent blank on a spectrophotometer[11]. This was compared with the color formed from an aliquot of a standard thimerosal solution (10 ml; 0.001% w/v) treated in an identical manner.

Preparation of Dithizone and Thimerosal Solutions—Dithizone (64 mg) was dissolved in chloroform (100 ml); 10 ml of this solution was diluted to 100 ml with chloroform. Thimerosal (100 mg) was dissolved in water (100 ml). Two successive dilutions of 10 ml to 100 ml were made to obtain the standard solution.

[1] BDH Chemicals Ltd., Poole, England.
[2] Cambrian Chemicals, Croydon, England.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Aldrich Chemical Co., Ltd., Gillingham, Dorset, England.
[5] Model CE 210, Cecil Instruments Ltd., Cambridge, England.
[6] Spherisorb 10 ODS, Phase Separations Ltd., Clwyd, Wales.
[7] Rheodyne Inc., Cotati, Calif.
[8] Model CE 212, Cecil Instruments Ltd., Cambridge, England.
[9] Silica gel 60 F_{254} (Merck) BDH Chemicals Ltd., Poole, England.
[10] $1P_5$, Whatman Lab. Sales Ltd., Maidstone, Kent, England.
[11] Model SP8-1000, Pye Unicam Ltd., Cambridge, England.

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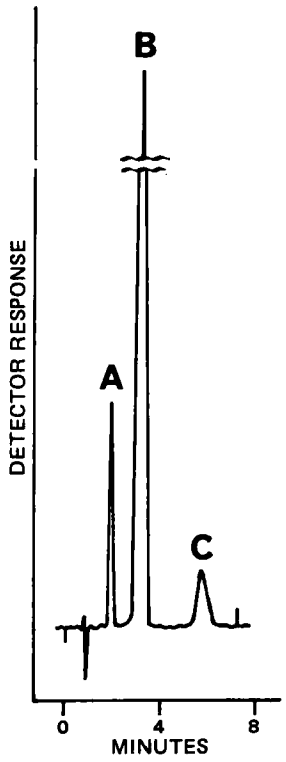


Figure 1—*Chromatogram of thiosalicylic acid (A), thimerosal (B), and 2,2′-dithiosalicylic acid (C).*

Hydrolysis of Thiomerosal in Aqueous Solution and in Saline—Solutions of thimerosal at a concentration of 0.5% in water and in 0.8% saline were heated under reflux and samples were removed at convenient intervals for TLC analysis. After 1 week, TLC (system B) showed one secondary spot at R_f 0.69 (nonabsorbing UV 254; yellow with dithizone) plus unreacted thimerosal (R_f 0.43). Neither solution contained any thimerosal after 3 weeks, but TLC now showed two major spots at R_f 0.23 and 0.30. The spot at R_f 0.69 was absent.

Both solutions were then cooled and acidified with hydrochloric acid to give white precipitates which were extracted into chloroform. The aqueous phases still contained insoluble materials which were filtered off, washed with water, and dried to give white solids, mp 283° (from the aqueous reaction) and 288° (from the saline reaction) identified as 2,2′-dithiosalicylic acid (IV) [lit. (2) mp 287–290°]. IR analysis gave an intense band at 1690 cm^{-1} due to the C=O stretching of an aromatic carboxylic acid. UV analysis showed two maxima at 252 and 313 nm in ethanol–0.1 *M* NaOH. TLC of the chloroform extracts showed two spots at R_f 0.23 and 0.30. Droplets of elemental mercury were also found in both solutions.

Stability in 0.8% Saline—*Thiosalicylic Acid*—A 0.2% solution of thiosalicylic acid in 0.8% saline was refluxed for 6 days. TLC (system B) showed complete conversion to IV (R_f 0.23); this was confirmed by UV analysis.

Ethylmercuric Chloride—A 0.2% solution/suspension of ethylmercuric chloride in 0.8% saline was refluxed for 6 days and monitored by TLC (system B). There was no indication of decomposition during this period and no visible formation of metallic mercury in the reaction vessel.

Interaction of Thiosalicylic Acid and Ethylmercuric Compounds in Aqueous Solution—Thiosalicylic acid (0.16 g, 1 mmole) and ethylmercuric chloride (0.27 g, 1 mmole) in water (20 ml) containing 1 *M* NaOH (1 ml) were refluxed for 1 week. TLC (system B) showed the formation of IV (R_f 0.23) and loss of ethylmercuric chloride (R_f 0.69). There was also deposition of metallic mercury. A parallel experiment with thiosalicylic acid (0.16 g, 1 mmole) and ethylmercuric hydroxide (0.25 g, 1 mmole) in 0.8% saline (20 ml) containing 1 *M* NaOH (1 ml) gave similar results.

RESULTS AND DISCUSSION

Decomposition Studies—The formation of II and III, or ethylmercuric chloride (V) in the presence of chloride ions, in the hydrolysis of I in water and 0.8% saline has been confirmed by TLC (Table I). No I and III (or V) remained after refluxing for 3 weeks, and the major products were shown to be II and IV by TLC and spectroscopic analysis. Mercury was also deposited from these solutions. The reaction shown in Scheme I represents the decomposition pathway of I under aqueous conditions. Compounds II and IV have been named and represented as the free carboxylic acids throughout, although in aqueous solution they will usually be present as their sodium salts.

Table I—R_f Values and Response to the Spray Reagent on TLC of I–V

Compound	R_f Values System A	R_f Values System B	Color with Dithizone
Thimerosal (I)	0.54	0.43	Yellow
Ethylmercuric chloride (V)	0.76	0.69	Lemon yellow
Ethylmercuric hydroxide (III)	0.70	0.65	Lemon yellow
Thiosalicylic acid (II)	0.40	0.29	Faint mauve[a]
2,2′-Dithiosalicylic acid (IV)	0.30	0.23	Faint mauve-pink[a]

[a] Color develops on standing.

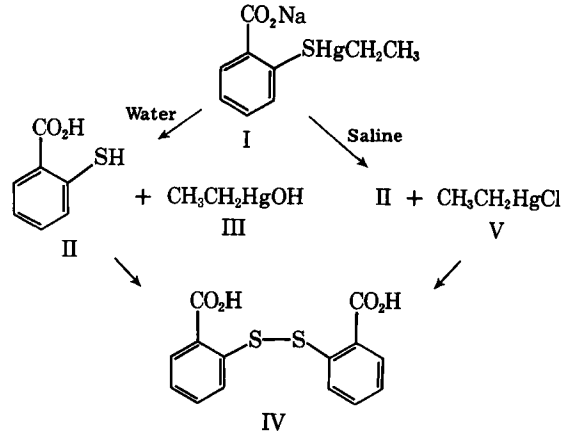
It has been demonstrated that II oxidized to IV on heating in 0.8% saline. Compound V alone, when heated in 0.8% saline, failed to degrade to mercury. However equimolar mixtures of II and III, and of II and V, have been shown to interact when heated in aqueous solution. As II was oxidized to IV, the ethylmercuric compounds (III and V) were correspondingly reduced to metallic mercury.

Analytical Studies—During the development of a reverse-phase HPLC method various water–methanol mixtures were tried and the pH of the eluant was controlled using phosphoric acid to reduce the effect of peak tailing. The eluant allowed monitoring at 222 nm, the UV absorption maximum of thimerosal at this pH. The mobile phase chosen for the analysis gave the following k' values: thiosalicylic acid, 1.5; thimerosal, 3.0; 2,2′-dithiosalicylic acid, 6.0. The effect of decreasing the proportion of methanol relative to water in the mobile phase was to increase the retention of all three components resulting in a high overall analysis time, while an increase in the methanol–water ratio impaired the resolution of thimerosal and thiosalicylic acid.

A standard calibration curve for thimerosal was constructed using a stock solution of 102 μg/ml and three solutions of 61.2, 40.8, and 20.4 μg/ml prepared by dilution of the stock solution. The $y = mx + c$ linear regression equation gave a slope of 1844, and intercept of 1577, and was linear in the range of 20.4–102 μg/ml (the corresponding integrator response ranging from 38,741 to 189,527). The plot gave a Pearson's correlation coefficient of 0.99998 ($p < 0.01$).

It was considered that the good fit and linearity of the calibration plot (which also passed very close to the origin) together with the good reproducibility of the method (coefficient of variation, 0.7%) allowed analysis to be carried out using drug peak areas alone and obviated the need for an internal standard. The limits of detection when 25-μl injections of solutions were made with a detector attenuation of 0.05 AUFS were 0.2 μg/ml for thiosalicylic acid and 0.1 μg/ml for thimerosal.

The HPLC procedure was used to assay thimerosal in samples of 0.01% aqueous solutions after storage in various containers for 24 months at



Scheme I—Reaction scheme for the decomposition of thimerosal in aqueous solution.

Table II—Results of HPLC and Dithizone Analyses of 0.01% Aqueous Thimerosal Solutions Stored for 2 Years at 5°, 25°, 37°, and 50° in Glass and Plastic Containers

Container	Temp, °	Recovery of Thimerosal, %				Thiosalicylic Acid, %			
		HPLC		Dithizone		Found		Calculated[a]	
		A[b]	B[b]	A	B	A	B	A	B
Glass ampules	5	100.5	97.6	99.6	98.1	4.8	3.4	0.0	2.4
	25	85.9	88.2	97.2	97.3	15.9	12.6	14.2	11.8
	37	80.4	80.4	95.4	94.5	20.8	18.9	19.7	19.7
	50	40.0	38.9	74.2	71.7	53.5	44.1	60.1	61.2
Glass ampules (autoclaved)	5	55.7	82.9	87.4	99.5	26.5	18.5	44.4	17.1
	25	66.5	63.8	89.5	97.7	24.6	24.1	33.6	36.2
	37	24.4	80.7	88.1	94.7	64.3	18.1	75.6	19.4
	50	9.3	29.1	59.7	74.9	56.2	49.3	90.6	70.9
Polyethylene bottles	5	95.1	99.6	95.8	100.0	5.5	3.8	5.0	0.5
	25	79.4	85.1	87.4	89.9	11.2	11.1	20.5	15.0
	37	67.3	65.1	76.5	69.1	15.0	16.8	32.8	34.9
	50	34.3	0.0	36.4	6.9	9.1	10.0	65.6	100.0
Polypropylene bottles	5	95.5		97.4		5.2		4.5	
	25	77.8		89.5		16.4		22.3	
	37	69.6		71.2		23.2		30.5	
	50	0.0		18.3		39.4		100.0	
Polyethylene containers	5	94.5		98.5		3.6		5.5	
	25	68.3		12.8		14.5		31.8	
	37	0.0		12.0		30.1		100.0	

[a] This figure represents the amount of thiosalicylic acid expected from the decomposition indicated by the HPLC assay of thimerosal. [b] A and B refer to assay values on two separate samples.

5°, 25°, 37°, and 50°. The containers included glass ampules, one-half of which were autoclaved at 120° for 20 min before the start of the storage test (the other half were not autoclaved) and three types of plastic containers. The results, together with those obtained colorimetrically using dithizone, are given in Table II.

After storage, the solutions in glass ampules that were not autoclaved showed HPLC assays lower than those obtained by the dithizone method. This trend was even more marked in those solutions which had been previously autoclaved. These figures are a reflection of the greater specificity of the HPLC method compared with the colorimetric assay.

The level of thimerosal after storage in plastic containers was found to be lower than that found in glass ampules especially at higher storage temperatures. There was, in general, fairly good agreement between the two assay methods for plastic containers. From the HPLC assay of solutions in plastic containers, it is clear that thimerosal is lost from solution by hydrolysis and by some form of sorption onto the containers; these effects increase with temperature. In plastic containers at 25° there was also less thiosalicylic acid detected than would have been anticipated from the thimerosal levels; this trend became more marked at 37° and 50°, providing a further indication of sorption of thimerosal onto the container. By comparison, in glass containers there was reasonably good mass balance between thimerosal decomposed and thiosalicylic acid formed up to 37°, less so at 50°. The sorption of organomercury compounds, including thimerosal, onto the surface of plastic containers has been noted previously (6, 18) and was shown to be dependent on storage temperature and time for a given plastic.

Table III—Results of the HPLC Assays of Thimerosal Solutions in Water and in Saline Stored for 15 days at 5°, 25°, and 50° in Glass Containers

Initial Thimerosal Concentration, %	Temp, °	Time, days	HPLC Assay of Thimerosal, %	
			Water	0.8% Saline
0.001	5	8	100.8	32.5
	25		101.8	<5
	50		28.9	<5
	5	15	105.2	30.3
	25		96.5	<5
	50		44.6	NA[a]
0.01	5	8	103.3	69.1
	25		99.7	50.0
	50		95.1	50.2
	5	15	95.6	69.2
	25		104.3	59.0
	50		NA[a]	NA[a]
0.1	5	8	104.7	98.6
	25		97.6	96.6
	50		99.9	92.0
	5	15	88.2	89.3
	25		79.4	72.8
	50		76.8	NA[a]

[a] NA = not analyzed.

Since ophthalmic pharmaceutical formulations are commonly rendered isotonic with the fluids in the eye using sodium chloride, the effect of this salt on the stability of thimerosal in aqueous solution was investigated. Thimerosal was stored as 0.1, 0.01, and 0.001% solutions in water and 0.8% saline at 5°, 25°, and 50° for up to 15 days. HPLC analysis was carried out after 8 and 15 days (Table III).

In water, thimerosal was stable except when stored at 50° at the lowest concentration (0.001%), the assay dropping to 30–40%. This effect of the initial concentration of thimerosal became even more marked in saline. After 8 days at 0.001% initial concentration, there was no thimerosal remaining at 25° and 50° and only ~30% at 5°. These figures present a disturbing picture, since they mirror what could be expected in ophthalmic formulations where thimerosal is very often present at concentrations of 0.001% in the presence of 0.8% saline. At 0.01 and 0.1% initial concentrations, sodium chloride had a less dramatic effect on the assay, but decomposition of thimerosal was still much faster in its presence.

In view of these findings on the effect of sodium chloride on the stability of thimerosal, equivalent pharmaceutical vehicles containing thimerosal at an initial concentration of 0.001% and 0.8% sodium chloride were prepared and stored at 5° for 3 months. HPLC assay showed no thimerosal (<10%) remaining, while the dithizone method gave satisfactory assay values of 101.3 and 102.3% of the labelled strength for two such samples. These figures further reflect the specificity of the HPLC method compared with the colorimetric procedure.

It was reported (5) that there was only 30% thimerosal remaining after storage in 0.4% aqueous sodium chloride, and in an equivalent pharmaceutical vehicle containing sodium chloride, for 2 months at 70°. However these findings were based on the semispecific UV or colorimetric methods for the assay of thimerosal and would seem to be somewhat optimistic. If the data in Table III together with that obtained from an equivalent pharmaceutical vehicle are considered, a much more pessimistic picture is presented of the stability of thimerosal in saline solution and, thus, in pharmaceutical preparations containing sodium chloride.

The exact reasons for the marked effect of sodium chloride on the decomposition of thimerosal are not clear at present. It is well known that chloride and other halides have a marked affinity to coordinate with mercury(II) compounds, and this could be a contributory factor in the detrimental effect of sodium chloride on the stability of thimerosal in solution. There is some evidence to show that the decomposition of thimerosal in water to thiosalicylic acid and ethylmercuric hydroxide is a reversible reaction. The effect of chloride could be to disturb this equilibrium by reaction with the ethylmercuric hydroxide to give ethylmercuric chloride, which does not react nearly as readily with thiosalicylic acid in neutral solution to give thimerosal.

Further investigations will be needed to substantiate this mechanism of the action of chloride ions and other anionic species. What is evident

from these investigations is that the use of sodium chloride as an isotonic agent with thimerosal as preservative in ophthalmic preparations is open to question.

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Bioavailability of Propylthiouracil in Humans

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Abstract □ Single lots of five commercially available 50-mg propylthiouracil formulations were evaluated *in vitro* and *in vivo*. Each product met the USP XIX specifications for drug content, content uniformity, and disintegration time. However, major differences were noted among products in their rate and extent of dissolution. Statistically significant differences ($p < 0.05$) were observed *in vivo* among the drug formulations at all but one of the sampling times, as determined from crossover blood level studies in 12 healthy male volunteers. The differences among the areas under the plasma level–time curves for the various products were not statistically significant. No statistically significant correlations were found between the *in vitro* and *in vivo* parameters studied.

Keyphrases □ Propylthiouracil—human plasma levels *in vivo*, bioavailability, correlation with *in vitro* dissolution □ Bioavailability—propylthiouracil, human plasma, correlation with *in vitro* dissolution □ Dissolution, *in vitro*—propylthiouracil, correlation with *in vivo* bioavailability, humans

Propylthiouracil, a thyrostatic drug that inhibits the synthesis of hormones within the thyroid gland and reduces the conversion of thyroxine (T_4) to the more potent triiodothyronine, T_3, in the peripheral tissues (1), is prescribed for the chronic treatment of hyperthyroidism and the preparation of hyperthyroid patients for surgery. While differences in bioavailability among propylthiouracil formulations have not been documented, propylthiouracil has been included in several lists of drugs with potential or actual differences in bioavailability (2, 3). The present study involved a crossover comparison to assess the relative bioavailability of five currently marketed products.

EXPERIMENTAL

Product Selection—Five single lots of 50-mg propylthiouracil tablets from separate manufacturers were evaluated; the individual products are identified in Table I. The sixth formulation, a solution of propylthiouracil, was utilized as a reference and was prepared as follows.

A stock solution of sucrose–glycerin was prepared by combining 6.5 g of sucrose with 12.5 ml of glycerin, diluting to 500 ml with distilled water. The day before each study a sucrose–glycerin–citric acid solution was prepared by adding 2.0 ml of 0.5 *M* citric acid to 95.4 ml of the sucrose–glycerin stock solution. On the day of each study the propylthiouracil reference solution was freshly prepared using USP propylthiouracil powder[1]. A 150-mg quantity of the powdered propylthiouracil was accurately weighed and dissolved in 6 ml of 0.2 *M* NaOH. The resulting solution was then diluted immediately with 24 ml of the sucrose–glycerin–citric acid solution and administered to the subject.

Clinical Study Protocol—Twelve male volunteers[2] underwent urinalysis and hematological and blood chemistry[3] testing to ensure that they were in good health. Also included in the initial medical evaluation was a T_4 determination by radioimmunoassay, T_3 uptake, and free thyroxine blood study. As a precaution against possible side effects of the propylthiouracil, the white blood cell and differential count, as well as prothrombin time, were monitored at the midpoint of the 6-week study. The subjects ranged in age from 20 to 25 years, in height from 172 to 198 cm, and in weight from 72 to 93 kg; all were considered to be of normal weight for their height (4).

The sequence of dose administration was based on a crossover matrix, designed to minimize the influence of any residual or cumulative effects of the preceding doses (5). Each subject received three 50-mg tablets or reference solution equivalent to 150 mg of propylthiouracil once a week for 6 weeks. The propylthiouracil formulations were administered with 200 ml of water in the morning following an overnight fast[4]. No food and water were permitted for 4 hr after ingestion of the dose. The subjects were instructed to avoid any food high in fat content on days of testing to minimize analytical problems associated with excessive lipids in the plasma. While the subjects were not sequestered on the days of testing they were instructed to avoid undue exercise. Subjects were also cautioned to avoid any other medication during the 6-week period of the study.

[1] USP propylthiouracil powder was provided by Lederle Labs.

[2] Staff and students of the University of Tennessee Center for the Health Sciences. Written informed consent was obtained.

[3] SMA 18/90.

[4] A standardized meal was not required prior to fasting.

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