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Radioimmunoassay for Danazol in Human and Monkey Plasma

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Abstract □ A sensitive method is described for the radioimmunoassay of danazol in monkey and human plasma. Antiserum was developed in rabbits, and a second antibody was used to separate bound from free danazol. The radioimmunoassay was specific for danazol, and the limit of detection ranged from 1.4 to 2.8 ng/ml. Exogenous danazol could be quantitated accurately in both monkey and human plasma. The radioimmunoassay results agreed with values obtained by inverse isotope dilution after intravenous administration of ^{14}C-danazol to monkeys. The assay was used successfully to measure danazol in plasma from human volunteers receiving 200 mg of danazol.

Keyphrases □ Danazol—radioimmunoassay in human and monkey plasma □ Radioimmunoassay—danazol in human and monkey plasma □ Anterior pituitary suppressants—danazol, radioimmunoassay in human and monkey plasma

Danazol[1] (17α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17-ol), a novel steroid, lacks estrogenic or progestational activity upon oral administration but has markedly attenuated androgenic potential (an "impeded" androgen) (1). It inhibits either pituitary gonadotropin synthesis or release in rodents (1, 2), monkeys (3), and humans (4). In males, it depresses the sperm count (5); in women, danazol relieves the symptomatology of endometriosis (6) and may be valuable in the management of benign breast disorders (7).

Limited studies have appeared on plasma danazol concentrations (8, 9). The present report describes a sensitive and accurate method for the measurement of danazol in plasma of both monkeys and humans.

EXPERIMENTAL

Preparation of Antidanazol Serum—The danazol hapten[2] was conjugated with ϵ-amino groups of lysine residues in bovine serum albumin by a carbodiimide condensation (10). A trinitrobenzenesulfonic acid determination (11) indicated that approximately nine hapten molecules were conjugated to each albumin. The hapten–protein conjugate (1.0–2.0 mg) was emulsified in complete Freund's adjuvant[3] and injected intradermally at multiple sites along the dorsal surfaces of three Dutch belted rabbits (12). Booster immunizations of the conjugate in incomplete Freund's adjuvant were administered at approximately 3-week intervals. The rabbits were bled *via* the marginal ear vein.

Preparation of Second Antibody—Anti-rabbit γ-globulin was prepared by repeated subcutaneous injection at monthly intervals of rabbit γ-globulin[4], 100 μg, emulsified in complete Freund's adjuvant to an adult goat. The goat was bled 2 weeks after each immunization. The dilution of the second antibody was 1:16, as determined by a progesterone assay[5] according to the procedure of Midgley *et al.* (13).

Solutions—Iodination buffer contained 0.5 *M* Na_2HPO_4–0.5 *M* NaH_2PO_4 (4:1), adjusted to a final pH of 7.5. Dilute iodination buffer was the same buffer diluted 1:10 with water. Phosphate-buffered saline was made by dissolving 24.51 g of sodium chloride, 1.77 g of monobasic sodium phosphate monohydrate, 3.21 g of dibasic sodium phosphate, and 0.285 g of thimerosal[6] in 3 liters of water and adjusting to pH 7.0. The assay buffer solution contained 0.1% (w/v) gelatin dissolved in phosphate-buffered saline.

Radioiodination—A methyl tyrosinate derivative of danazol[7] (I) was radioiodinated at room temperature by a modification of the method of Greenwood and Hunter (14). Iodination buffer (50 μl) was mixed with 5 μl of a methanol solution of I (1 mg/ml) in a vial. Sodium ^{125}I-iodide[8] (1.0 mCi) was added, the vial was stoppered, and the contents were mixed gently. Chloramine-T[9], 30 μg in 15 μl of dilute iodination buffer, was added, and the reaction mixture was agitated for 2 min. The reaction was stopped by addition of 60 μg of sodium metabisulfite in 30 μl of dilute iodination buffer.

Transfer solution (100 μl), containing 16% (w/v) sucrose dissolved in the assay buffer solution, was added, and the contents of the vial were layered on a 0.5 × 20-cm anion exchange[10] column equilibrated with the assay buffer solution. The vial was rinsed with 70 μl of a solution containing 8% (w/v) sucrose dissolved in the assay buffer, which was then added to the column. The column eluate was collected in 1.0-ml fractions, which were counted to determine the radioactivity profile.

Extraction Procedure—A methanol solution containing 20 μg of hydrocortisone (2 mg/ml) and 40,000 dpm of 1,2-^{3}H-progesterone[11] (40 Ci/mmole) was added to a set of 15 × 85-mm disposable glass tubes, and the solvent was evaporated in a 45° water bath under a nitrogen stream. Plasma (0.5 ml), containing known or unknown amounts of danazol, and water (0.5 ml) were added to the tubes and they were mixed. The mixture was allowed to remain either at room temperature for 2 hr or at 4° overnight. Then *n*-hexane[12] (5 ml) was added to each tube, and the tubes were shaken mechanically for 5 min.

[1] Danocrine, Winthrop Laboratories, New York, N.Y.

[2] 17-Hydroxy-17α-pregn-4-en-20-yno[2,3-*d*]isoxazol-6-ylideneaminoxyacetic acid.

[3] Difco Laboratories, Detroit, Mich.

[4] Fraction II, Miles Laboratories, Kankakee, Ill.

[5] J. E. Peterson and G. D. Niswender, unpublished data.

[6] Sigma Chemical Co., St. Louis, Mo.

[7] Methyl 4-hydroxy-α-[[[[[(17α)-17-hydroxypregna-2,4-dien-20-yno[2,3-*d*]-isoxazol-6-ylidene]amino]oxy]acetyl]amino]benzenepropanoate.

[8] New England Nuclear, Boston, Mass.

[9] Eastman Kodak, Rochester, N.Y.

[10] QAE-Sephadex, Q-25-120, Sigma Chemical Co., St. Louis, Mo.

[11] New England Nuclear, Boston, Mass.

[12] Nanograde, Mallinckrodt, St. Louis, Mo.

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Table I—Relative Affinities of Danazol, Related Compounds, and Native Steroids to the Antiserum

Compound	Relative Affinity
Danazol	1.00
Ethisterone (17-hydroxy-17α-pregn-4-en-20-yn-3-one)	0.055
2-Aminomethylene-17-hydroxy-17α-pregn-4-en-20-yn-3-one	0.031
17-Hydroxy-2α-hydroxymethyl-17α-pregn-4-en-20-yn-3-one	<0.031
17-Hydroxy-3-oxo-17α-pregna-1,4-dien-20-yne-2-carboxyaldehyde	<0.015
6β,17-Dihydroxy-17α-pregn-4-en-20-yn-3-one	<0.015
17-Hydroxy-2-hydroxymethyl-17α-pregna-1,4-dien-20-yn-3-one	<0.015
6β,17-Dihydroxy-2α-hydroxymethyl-17α-pregn-4-en-20-yn-3-one	<0.003
17α-Pregn-4-en-20-yno[2,3-*d*]isoxazol-6β,11β,17-triol	0.001
Testosterone (17β-hydroxy-4-androsten-3-one)	0.004
Progesterone (4-pregnene-3,20-dione)	<0.004
Estradiol [1,3,5(10)-estratriene-3,17β-diol]	<0.004
Androstenedione (4-androstene-3,17-dione)	<0.004
Hydrocortisone (11β,17,21-trihydroxy-4-pregnene-3,20-dione)	<0.006

The aqueous phase of each sample was frozen in a dry ice–acetone bath, and the hexane phase was decanted into another tube. The hexane extraction was repeated two additional times. The combined hexane extracts were evaporated to approximately 5 ml, and 1 ml of distilled water was added to the tubes. The tubes were mixed for 1 min, the aqueous phase was frozen in dry ice–acetone, and the hexane was decanted into a clean set of 15 × 85-mm tubes. Then the hexane was evaporated to dryness, and 20 μl of absolute alcohol was added to each tube.

The tubes were shaken, and 1 ml of assay buffer was added. Then the tubes were mixed, covered, and allowed to remain overnight at 4°. Recovery of ^{3}H-progesterone was determined by counting 300 μl of this solution and used to estimate the extraction efficiency of danazol.

Radioimmunoassay—The radioimmunoassay for danazol was similar to assays previously reported for other steroids (15). Duplicate samples of the plasma extracts at 100 and 200 μl were added to disposable culture tubes (12 × 75 mm). The samples were diluted to 500 μl with assay buffer; 200 μl of antidanazol serum, at a dilution of 1:2000 in nonimmune normal rabbit serum, was then added. Radioiodinated I, 100 μl, prepared by diluting the eluate from the ion-exchange column with assay buffer to 25,000–30,000 cpm/100 μl, was added.

All assay tubes were incubated for 4–6 hr at 4°, followed by addition of 200 μl of appropriately diluted second antibody. Incubation was continued for an additional 12–18 hr. At the end of the incubation period, 2.5 ml of cold phosphate-buffered saline was added, and the tubes were centrifuged at 2500 rpm for 30 min; the supernates were decanted, and the precipitates were counted. Duplicate standards with 0.1–100 ng of danazol were included in each assay.

Human Study—Ten healthy female volunteers received a 200-mg danazol capsule orally following an overnight fast. Blood samples (5 ml) were drawn into an oxalated tube at 0, 1, 2, 4, 6, 8, 12, 16, 20, and 24 hr. The blood samples were centrifuged promptly to separate the plasma, which was frozen until analyzed.

Monkey Study—An intravenous dose was prepared by dissolving 6.0 mg of ^{14}C-danazol[13] in absolute ethanol–physiological saline (1:1) to a final specific activity of 1.2×10^8 dpm/ml (1.37 mg/ml). Three rhesus monkeys received approximately 1 ml of this solution *via* the saphenous vein at a dose level of 0.31 mg/kg. Blood samples were removed from the femoral vein at intervals from 5 min to 3 hr. The samples were immediately centrifuged, and plasma was obtained. The plasma samples were frozen until assayed.

Inverse Isotope Dilution Technique—A measured amount (100–500 μl) of each monkey plasma sample was added to 1 ml of water, followed by 20 μg of nonradioactive danazol dissolved in chloroform (1 mg/ml). Each sample was extracted with hexane (2 × 10 ml), and the organic layer was separated from the aqueous phase as already described. The combined hexane extracts were evaporated to dryness under air, and the residue was dissolved in 50 μl of chloroform. The entire solution was injected into a liquid chromatograph using the following conditions: column, 5 μm silica[14] (4.2 × 150 mm); pressure, 500 psi; mobile phase, 3% (v/v) 2-propanol in redistilled pentane; and detector, UV, 254 nm.

The danazol fraction of each sample was collected and evaporated under air, and the residue was dissolved in 2 ml of methanol. A 1-ml aliquot was transferred to a counting vial containing 10 ml of scintillation fluid[15] and counted. The remaining methanol was evaporated to dryness under air. The residue was dissolved in 50 μl of chloroform and reinjected into the liquid chromatograph, using conditions similar to those described, with chloroform as the mobile phase. The danazol fraction of each sample was collected in a counting vial and evaporated. Methanol (1 ml) and 10 ml of scintillation fluid were added to the residue, and the solutions were counted.

Several blank samples were collected between injections and similarly counted. The average of these blanks was taken as the background counting rate for a given series of injections.

A set of direct standards was prepared in chloroform such that a 10-μl sample of each would contain an amount of danazol varying from 0.1 to 20 μg. These standards were injected onto the liquid chromatograph, and a standard curve was prepared prior to the injection of each set of experimental samples. Danazol recovery in each experimental sample was quantitated, using peak height, with reference to the standard curve.

The specific activity (disintegrations per minute per microgram) of the danazol in the second injection of each sample was calculated and used to determine the number of disintegrations per minute of intact danazol per milliliter of monkey plasma. The specific activity of the dose of danazol was then used to convert to nanograms per milliliter.

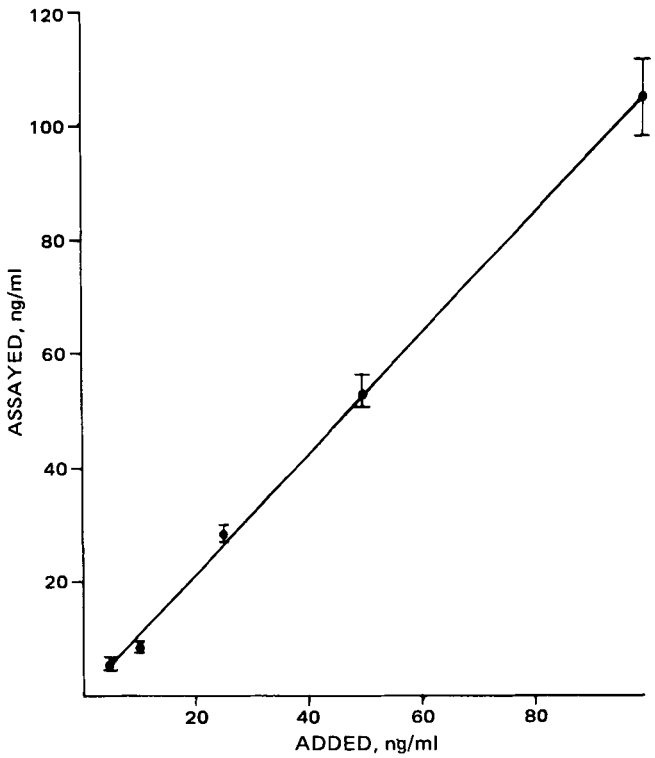

Figure 1—*Correlation of exogenous danazol added to human plasma with mean values obtained by radioimmunoassay. Slope = 1.055, intercept = 0.069, and* r *(correlation coefficient) = 0.999. The vertical line represents 2* SE (n = *8).*

RESULTS AND DISCUSSION

All three rabbits produced antiserums within 2 months that bound radioiodinated I at an initial dilution (200 μl) of 1:400 or greater. The dilution required to bind 40–50% of the radioactive I was 1:2000, equivalent to a 1:10,000 final dilution in the assay tube.

[13] Lot 61004, 13.4 mCi/mmole, Pathfinder Labs, St. Louis, Mo.

[14] Lichrosorb, E. M. Laboratories, Elmsford, N.Y.

[15] Biofluor, New England Nuclear, Boston, Mass.

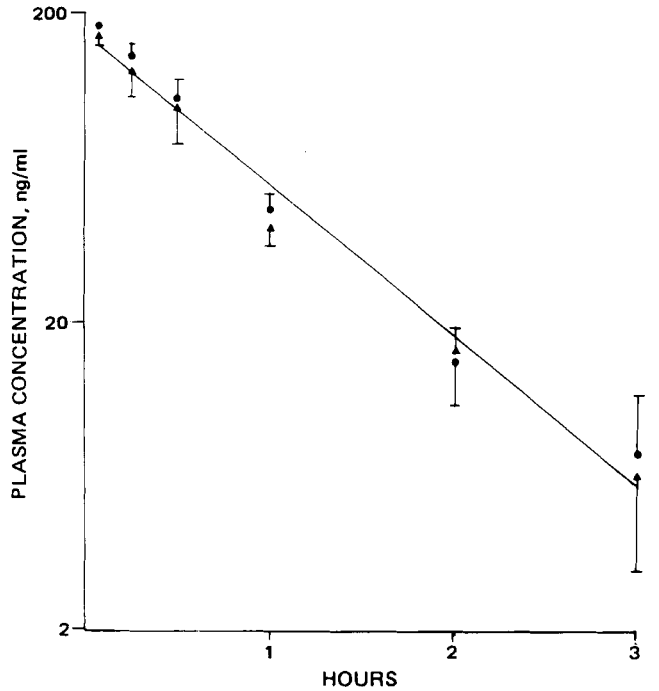

Figure 2—*Mean concentration of danazol in monkey plasma after 0.31 mg of danazol/kg iv. Key: ●, values obtained by radioimmunoassay; and ▲, values obtained by the inverse isotope dilution technique. The vertical line represents 1* SE *(n = 3).*

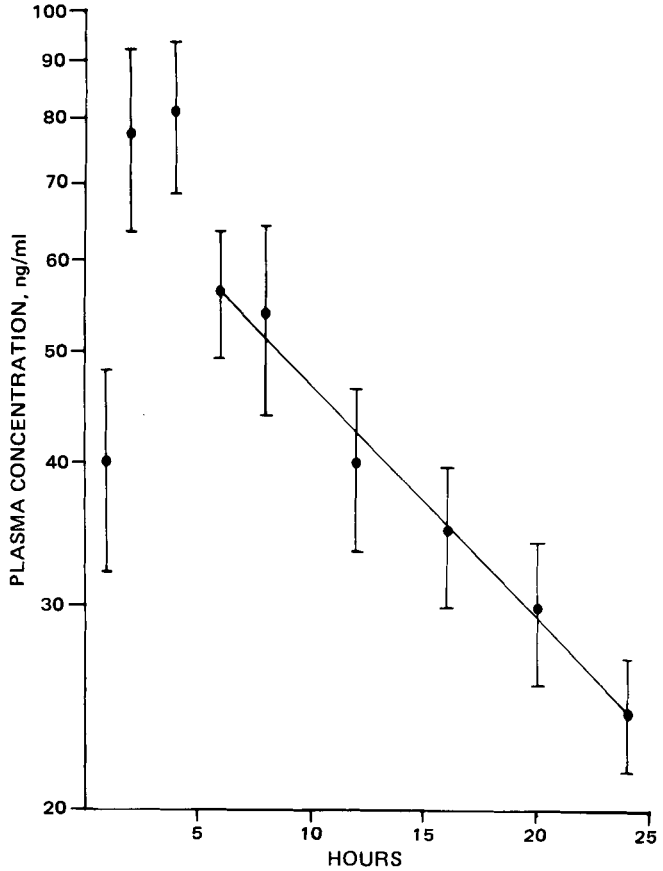

Figure 3—*Mean danazol concentration in plasma of women who received a single 200-mg capsule of danazol. The vertical line represents 2* SE *(n = 10).*

The specificity of antiserums from three rabbits on three different bleeding dates was assessed by determining the ability of naturally occurring steroids and compounds related to danazol (16) to compete with radioiodinated I for binding to the antibody. The amounts of each steroid required for 50% displacement of radioactivity were determined by comparison with the danazol standard curves to arrive at a relative affinity (17). The antiserum from one bleeding, exhibiting the least cross reactivity, was selected for all subsequent work. The relative affinities of potentially competitive steroids with this antiserum are shown in Table I. The only tested steroid to show significant binding activity was ethisterone (5.5%).

Sensitivity is defined as the smallest amount of steroid that can be measured in an aliquot of biological fluid. Sensitivity, S, may be expressed as:

$$S = \frac{2SD}{RF} \times 100 \qquad \text{(Eq. 1)}$$

where R is the fraction recovered, F is the fraction of the extracted steroid used in the assay, and SD is the standard deviation of the mean of the zero-time plasmas (18). The mean recovery, as estimated from ^{3}H-progesterone extraction efficiency, was 73.4 ± 0.63% (n = 88) from human plasma and 71.2 ± 0.46% (n = 18) from monkey plasma. The extraction properties of danazol and progesterone are similar; the mean recovery of ^{14}C-danazol from human plasma was 73.9 ± 0.35% (n = 6). The human plasma blank values gave a limit of detection of 1.40 ng/ml for the 100-μl aliquot and of 1.42 ng/ml for the 200-μl aliquot. The monkey plasma blank values gave 2.81 and 2.60 ng/ml for the 100 and 200-μl aliquots, respectively.

The within- and between-assay precision was evaluated by duplicate measurements of identical samples in the same assay and in replicate assays. The coefficient of variation, CV, can be estimated using (19):

$$CV = \sqrt{\frac{\sum d^2}{2n}} \qquad \text{(Eq. 2)}$$

where:

$$d = \frac{\text{highest value of each duplicate}}{\text{lowest value of each duplicate}} - 1 \times 100 \qquad \text{(Eq. 3)}$$

and n is the number of duplicate determinations.

The within-assay coefficient of variation ranged from 5.7 to 15.1% with a mean of 9.6%. The between-assay coefficient of variation ranged from 8.7 to 23.3% with a mean of 17.6%. There were no differences between monkey and human plasmas. These values are within the range reported for other steroid assays (17).

Exogenous danazol was quantitated accurately when varying amounts were added to human plasma, extracted, and assayed in the danazol radioimmunoassay (Fig. 1). A similar correlation was found for monkey plasma: slope, 0.981; intercept, 3.91; and correlation coefficient, 0.999. A comparison of the results of the radioimmunoassay with values obtained by the inverse isotope dilution technique on plasma from three monkeys that had been injected with ^{14}C-danazol showed excellent agreement (Fig. 2). The apparent first-order elimination half-life for the first 3 hr was 37 min.

The mean concentration of danazol in the plasma of 10 female volunteers receiving 200 mg of danazol is shown in Fig. 3. The apparent first-order elimination half-life of danazol was 14.7 hr as determined by least-squares regression on the data points of the terminal phase.

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Simulated Respiratory System for *In Vitro* Evaluation of Two Inhalation Delivery Systems Using Selected Steroids

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Received November 29, 1976, from the *Arnold and Marie Schwartz College of Pharmacy and Health Sciences of Long Island University, Brooklyn, NY 11201.* Accepted for publication February 7, 1978.

Abstract □ A simulated respiratory system was developed for the *in vitro* evaluation of two differently designed oral inhalation delivery systems. The deposition properties of a newly designed delivery system used for triamcinolone acetonide were compared to the more conventional, commercially available adapter utilized for an aerosol containing beclomethasone dipropionate. The simulated respiratory system was constructed so that the delivered dose of active ingredient could be classified into two fractions: the fraction that would be deposited in the oral cavity and throat and the fraction that would reach the desired site of activity in the respiratory tract. Based on this method, the newly designed system delivered more than 95% of the labeled dose to the desired site. The beclomethasone dipropionate aerosol system, which was observed to discharge the active ingredient with a greater intensity, delivered approximately 40% of the labeled dose. The particle-size distribution of the dose dispensed from the newly designed delivery system attached to the triamcinolone acetonide aerosol was determined using an impactor technique. No effort was made to correlate these results with an *in vivo* response.

Keyphrases □ Respiratory system, simulated—developed for *in vitro* evaluation of inhalation delivery systems □ Delivery systems, inhalation—evaluated *in vitro* using simulated respiratory system □ Inhalation delivery systems—evaluated *in vitro* using simulated respiratory system

Oral aerosol products used by inhalation are intended to deliver the active ingredient in the desired particle-size range to the proper portion of the respiratory system. In many instances, however, a fairly high percentage of formulation is retained in the oral adapter, mouth, and back of the throat instead of obtaining the desired deep penetration into the bronchi. This problem was discussed by Hayton (1). Since this loss is fairly consistent, many commonly used aerosol products contain an increased amount of active ingredient in the formulation so that the proper dose is delivered to the desired site of activity.

Various methods are useful for determining the dose of an aerosol delivered through an oral adapter in the desired portions of the lungs. Karig *et al.* (2) developed a compartmentalized lung chamber that could simulate different sized lung bronchi. They established an *in vitro* method for evaluating the penetration of solid particles in the lungs using several different aerosol generators. An air flow rate, based on a vacuum of 30.4 cm Hg, could be used to evaluate several different types of aerosols. The chamber size was based on literature values for the size of the human respiratory tract. Both solution- and suspension-type aerosols were studied, and it was concluded that it was possible to compare solution aerosols to suspension aerosols and to determine differences in their deposition.

The purpose of this study was to develop a suitable method for evaluating various oral adapters. In particular, the method should be capable of classifying the dispensed dose into a fraction that would be trapped in the oral cavity (mouth) and throat and a fraction that would reach the lungs. An artificial or simulated respiratory system was designed and constructed. A newly developed, different type of oral adapter, which may be capable of delivering a greater percentage of the active ingredient to the desired site, was attached to an aerosol product containing triamcinolone acetonide as the model drug and evaluated using the simulated respiratory system. For comparison, an aerosol product using a conventional oral adapter was also evaluated using the simulated respiratory system.

EXPERIMENTAL

Design of Simulated Respiratory System—The simulated respiratory system (Fig. 1) was made of glass and was scaled to twice the relative dimensions of a normal respiratory system. It was designed to follow the normal structure of the respiratory system. The entrance to the simulated respiratory system opened into an area similar to the oral cavity. This area then opened into three separate chambers: the chamber above the opening represented the nasal passageway; another opening, below and to the left, represented the esophagus; and the third opening represented the entrance to the lungs. Removable ground-glass stoppers were fitted at the opening located at the base of the unit (esophagus) and to the simulated nasal passageway.

The simulated respiratory system was then fitted to a modified particle-size impactor[1] capable of classifying particles from 0.5 to 32 μm. However, the six stages of the impactor were removed, and only the last stage (0.5-μm size) was used. The portion of the simulated respiratory system representing the opening to the lungs was fitted to the opening of the impactor, and a vacuum was applied through this "lung" opening.

[1] Cascade impactor, model CI-S-6, Scientific Advances, Columbus, Ohio.

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