CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Capsule size 2

D-(+)-Lactose monohydrate (Fluka®, Switzerland)

Distilled water

Methanol HPLC grade (J.T.Baker, N.J.,USA)

Micronized salbutamol sulfate (DDSA Pharmaceutical, London, UK)

Polyamide membrane 0.45 µm (Sartorius, Goetting, Germany)

3.2 Equipments

Andersen Cascade Impactor (Atlanta, Goergia, USA)

Blister patch maker (made in house)

Filter apparatus (Millipore, USA)

Laser diffractometer (Mastersizer, Malvern, UK)

Luminescence spectrometer LS50B (Perkin Elmer, USA)

Magnetic stirrer (Framo®-Geratetechnik, Germany)

Manometer (Extech, USA)

Planetary MonoMill pulverisette 6 (Fritsch. GmbH, Idar-Oberstein, Germany)

Pressure drop apparatus (made in house)

Scanning Electron Microscope (Jeol, Japan) Scanning electron microscopy instruments: Scanning electron microscope, JSM-5800LV (JEOL Ltd., Tokyo, Japan) and Electron probe micro analyzer, JEOL JXA-880R (JEOL Ltd., Tokyo, Japan) equipped with an energy dispersive spectrometry analyzer, Oxford ISIS 300 (Isis Innovation Ltd., Oxford, UK).

Sieve shaker (Fritsch GmbH, Idar-Oberstein, Germany)

Sonicator (Elma, Germany)

V-shape mixer (Superline, Japan)

Vacuum pump (Gast, USA)

Vernier caliper (Kanon, Japan)

3.3 Validation of spectrofluorometry method

Model drug in this study is Micronized salbutamol sulfate. Dry powder formulations used in this study contain lactose as a carrier with drug particles adhered on surface carrier. Amount of drug deposition on each stage of Andersen Cascade Impactor (ACI) is generally in µg amount. The determination technique must have sensitivity to detect small amount of salbutamol sulfate and eliminate interference from the formulation ingredients. The UV analytical method is not sensitive enough to analyze drug in low µg level and cannot eliminate interferences from the lactose (Beilmann *et al*., 2006). Nevertheless, a separation technique, i.e. HPLC-UV is time consuming compared to non-seperating technique. The spectrofluorometry technique has reported that sensitivity met requirments to determine salbutamol sulfate and no interferences were found from lactose (Srichana et al. 2003). For large numbers of experiment set like in this study, the spectrofluorometry was employed as a tool for drug analysis. To ensure that the analytical method is precise and

reliable, in this study, the method for the determination of salbutamol sulfate was validated prior to formulation of dry powder inhalers.

3.3.1 Spectrofluorometry conditions

Fluorometric experiments were performed using Luminescence spectrometer LS50B (Perkin Elmer). The quartz cuvette (1x1 cm) was used in this study. The Fluorescence technique in this study was adapted from previous study by Srichana et al. (2003). Quartz cuvette filled with salbutamol sulfate solution in distilled water was excited with wavelength at 219 nm and then measured the luminescence intensity at 310 nm. Both slit width used in this method was 3.7 nm. Distilled water was used as a blank.

3.3.2 Calibration curve for standard salbutamol sulfate

Stock solutions of salbutamol sulfate was prepared by dissolving 10 mg of drug in water. Then volume was completed to 100 ml with the same solvent. The stock solution was kept in the refrigerator and protected from light until use.

The salbutamol sulfate stock solution was diluted stepwise with water to give a final drug concentrations of 0.2, 0.6, 1.0, 1.6, 2.0 µg/ml. Then, final concentrations were determined by spectrofluorometry as previously described. Calibration curve was generated by plotting the corresponding concentrations of the drug versus fluorescence intensity.

The linearity response of salbutamol sulfate was determined by analyzing corresponding intensity 5 times for each concentration in range of 0.2-2 µg/ml. The linearity was determined by calculating the correlation coefficient value (R^2).

3.3.3 Method validation of salbutamol sulfate

A stock solution was prepared by weighing 10 mg of salbutamol sulfate and dissolved in distilled water to 100 ml in volumetric flask. The stock solution was diluted stepwise with distilled water to obtain concentrations of 0.2, 1.0, 2.0 µg/ml.

The intra-day precision data obtained from 5 repeated analyses of a preparation on the same day. The inter-day precision data obtained by analyzing freshly prepared samples of 2 determinations per day within five days. The precision was determined within 3 concentrations at 0.2, 1.0 and 2.0 µg/ml by calculating the standard deviation (SD) of analyte intensity over the mean intensity ($\bar{x}$) at each concentration. A percentage of the relative standard deviation (%RSD) was obtained from (SD/$\bar{x}$) x100. This value should be less than 2% (USP 2002).

The accuracy of an analysis was determined by the systemic error involved. It was determined by calculating the recovery of the analyte at concentration 0.2, 1.0 and 2.0 µg/ml. Recovery was evaluated by comparing the theoretical and measured concentration of an analyte.

3.4 Formulation design and devices evaluation

Because of different carrier sizes in the DPI formulation provides different adhesive force of drug on the carrier. In previous studies, while carrier particle sizes was varied, it showed that lactose carrier size about 35 µm provided optimal %FPF (Louey et al. 2003). In this study, three ranges of the carrier size between 20-90 µm were collected by sieving method to give different carrier sizes to prepare three different formulations.

The device resistance also is an important factor to consider in device design. In previous study, the high specific resistance devices would be expected to generate higher turbulence air-flow and increasing of the %FPF when operating with higher air flow-rate (Srichana et al, 1998). However, the fact is high of device resistance affects patient's inhalation effort, resulting in lower inhalation flow-rate. The most favor method to determine device resistance is to measure pressure drop across device. In this study, the pressure drop across device apparatus was adapted from apparatus designed by Novartis, Horsham, UK.

3.4.1 Preparation of the micronized lactose carrier

Lactose carrier was dried at 50 °C for 24 hours in a tray drier. Then, lactose carrier was reduced in size by a Ball mill at 350 rpm for 1 h to obtain micronized particles (particle size of lactose carrier in range of about 10-90 μm). Milled carrier particles were separated by size with air-blow through sieve series having open diameter of 90, 71, 30 and 20 μm. The collected size ranges of the carrier in this study are from 71-90, 30-71 and 20-30 μm. These three size ranges of lactose carrier were used to prepare 3 formulations having different drug delivery efficiency. All of powders were placed in a desiccator containing silica gel at room temperature until required for future use.

3.4.2 Particle size distribution measurement

Carrier sizes were determined by Laser diffraction technique using an independent particle size model fitted with a 100 mm lens. Light from a laser is shone into a cloud of particles, which are suspended in a liquid medium. The particles scatter the light, smaller particles scattering the light at larger angles than bigger particles. The scattered light can be measured by a series of photodetectors placed at different angles.

In this study, dispersion medium was prepared by dissolving saturated lactose in methanol for 1 night. Filtered saturated lactose solution with 0.22 μm membrane filter was used to prevent dissolve of lactose particle sampler while measuring and was measured as a background. The ultrasonication was used to prevent aggregate particles formation while operating. Background measurement was taken and then the sample was added and mixed homogeneously with the medium when the %obscuration was controlled at 10. In this study, five measurements were carried out for each sample.

3.4.3 Characterization of particle morphology

Visualization of particle surfaces of drug and carrier was observed under scanning electron microscope (SEM) technique. A small amount of each sample was scattered on an aluminum stub, the latter surface covered with clear double-sided adhesive tape. In order to obtain uniformly scattered samples the aluminum stub was tapped gently on its edge with a spatula. The particles were then coated with a 15 to 20 nm layer of gold using a sputter coater on an argon atmosphere (50 Pa) at 50 mA for 50 seconds. All formulations were stored in a desiccator at room temperature over silica gel.

3.4.4 Preparation of formulations

The formulation was prepared by mixing salbutamol sulphate with lactose at a ratio of 1:67.5. Each formulation contained 20 mg of drug and 1.35 g of each carrier in vial. Hand mixing was done for 10 min, then mixed with v-shape mixer for 2 h. Prepared formulation is equal to 50 DPI dose (1 dose = 27.4 mg). Formulation dosages were kept in desiccator and divided into 27.4 mg/dose (400 μg drug/dose), filled into either No.2 capsule for use with 3 commercial devices or blister pack for use with pipe devices.

3.4.5 Content uniformity of the formulation

Homogeneity of powder blends is required for DPIs. Normally the content uniformity of dosage is performed by sampling 10 areas per container. It is acceptable content when the %RSD is less than 6 (USP 2002). However, in this study, the formulation was prepared in a small container which is likely to obtain uniform mixture. Also, the number of dose is limited, thus content uniformity in this was done by random sampling powder of 5 samples in 3 areas; on top, in the middle and at the bottom of container.

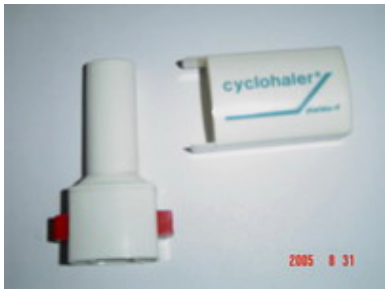
In formulations of salbutamol sulfate, 30 mg of powder blend was accurately weighed and dissolved by distilled water to complete to 100 ml within a volumetric flask. The 100 ml of this solution was further diluted by pipetting 5 ml aliquot and transferred to 25 ml volumetric flask and completed with distilled water again.

3.4.6 Dimension of devices

To evaluate physical properties between devices. Two different shapes of pipes were chosen; straight pipe (SANDA-SD 381, Hongkong) and curved pipe (SANDA-SD 101, Hongkong). Three commercial devices were compared; Spinhaler, Cyclohaler and Inhalator. These commercial devices were classified as low (Spinhaler), medium (Cyclohaler) and high (Inhalater) device resistance respectively as previously reported (Srichana et al, 1998). All of devices in this study are shown in Figure 3.1. Volume of device was measured by replacement of water into the mouthpiece, internal diameter and device dimension were measured with Vernier caliper and by devices cross section. Obtained data were used to compare differences between devices.

Spinhaler® (Fisons, USA)

SANDA-SD 381 (straight pipe)



Cyclohaler® (Pharbita, Netherlands)

SANDA-SD 101 (curved pipe)

Inhalator Ingelheim® (Boerhinger Ingelheim, Germany)

Figure 3.1 Dry powder inhaler devices used in this study

3.4.7 Device resistance measurement

The internal resistance of the DPI device can be determined by using an apparatus made in house (Figure3.2). The apparatus consisted of a new designed metal box, approximately 10x10x15 cm for use with long pipe devices, with a rubber polymer cover housing the device. The device was held

with rubber ring seals. Air was drawn through the apparatus at the selected flow-rate as determined by an air flow rotameter. The pressure drop across the apparatus containing the device was recorded by a digital manometer. Pressure drop measured between flow-rate of 20-90 L min^{-1}, the possibility operable maximum flow-rate are upon each type device. The device resistance is directly related with the pressure drop across device as shown in Figure 3.3. The square root of difference in pressure drop across device is equal to specific device resistance multiply with a flow-rate (Equation 3.1). More pressure drop causes a high resistance of the device. From experimental data, the device resistance can be calculated from slope of square root pressure drop plot with flow-rate.

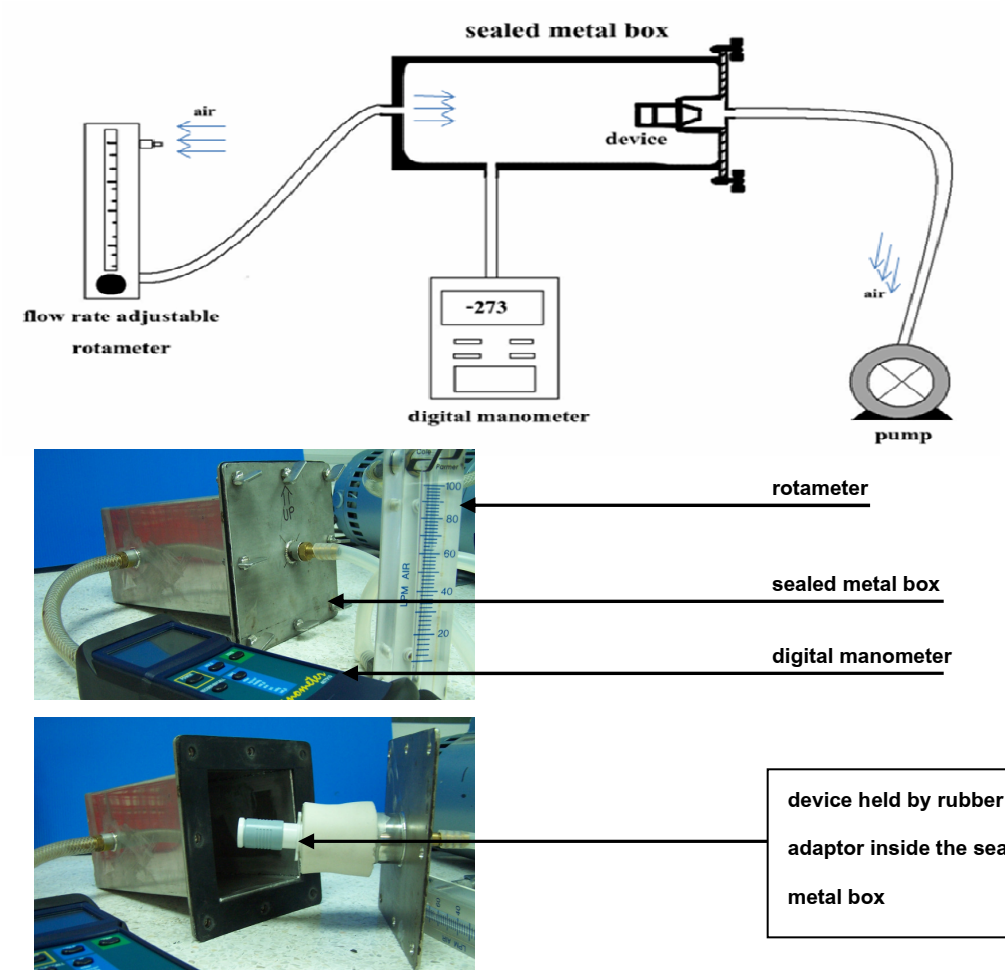


Figure 3.2 Pressure drop determining apparatus

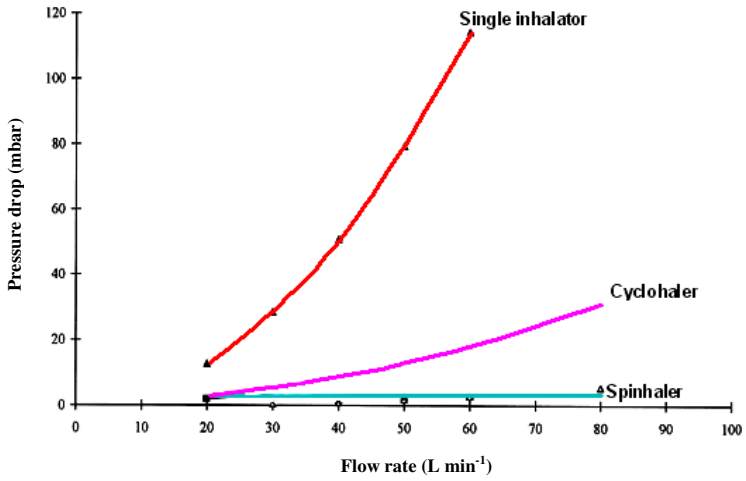


Figure 3.3 Relationships between pressure drop and flow-rate (adapted from Srichana, *et al*., 1998)

$$\sqrt{\Delta P_D} = R_D Q$$

P_D = the pressure drop across the device (mbar)

R_D = the specific resistance of the device (mbar)1/2/(L min^{-1})

Q = flow rate (L min^{-1})

Equation 3.1 Relationship between device resistance and pressure drop

The curves were constructed by plotting the square root of pressure drop versus the flow-rate. Slope from the graph is specific resistance of the device (R_D).

3.5 Devices performance evaluation

3.5.1 Drug deposition studies

Drug delivery was evaluated by eight stages Andersen Cascade Impactor (ACI; United States Pharmacopoeia, 1999), which was operated at 30, 60 and 90 L min^{-1} for 20, 10 and 6.7 s, respectively. This work out to be the same air volume drawn through each device. Drug retaining on each collection plate in each stage was eluted with water. Adjust volume to obtain appropriate concentration. Quantitative analysis was carried out by fluorescence technique. %Deposition was calculated on each stage. %Fine Particle Fraction (%FPF) and Mass Median Aerodynamic Diameter (MMAD) were used to determine drug delivery performance. %Emission (%EM) was calculated from percentage of drug release from device. %FPF was calculated from summation of amount of drug retained on stage at cut-off diameter below 5 μm. The stage cut-off diameter can be obtained from ACI validation. For operating flow rate at 30 L

min^{-1}, %FPF was calculated from stage 2-7 while operating with flow rate 60 L min^{-1} the FPF was obtained from stage 1-7 and when operating with flow rate 90 L min^{-1} the FPF was obtained from stage 0-7 (Figure 3.4).

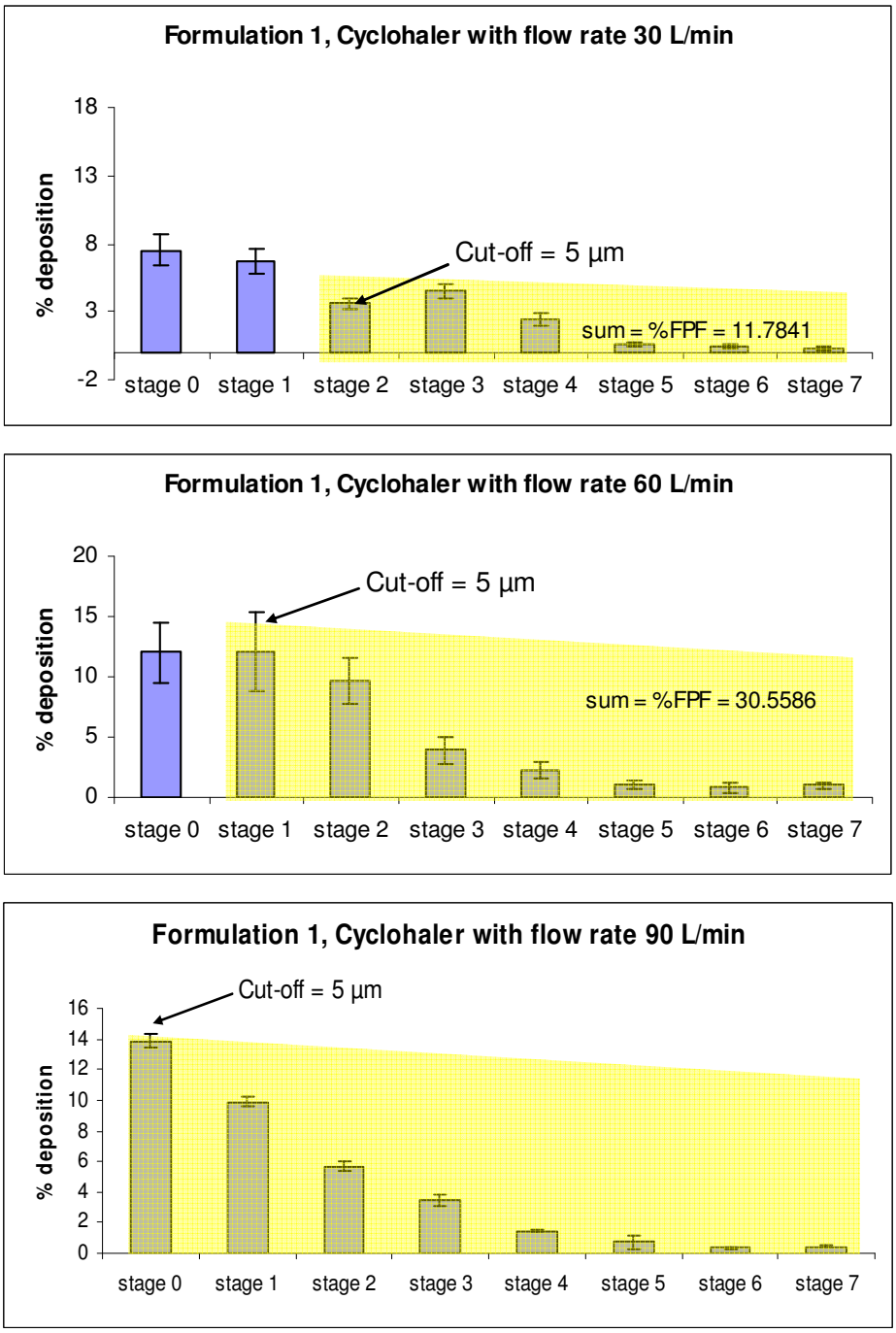


Figure 3.4 Calculating of %FPF at flow rate 30, 60 and 90 L min^{-1}

Figure 3.5 shows that MMAD obtained from median diameter of relative graph plot between log size cut-off diameter with z-value of drug cumulative percentage. The log size cut-off diameter obtained from well known cut-off diameter on every stages of ACI at each flow-rate revaluated to log scale. The cumulative drug deposition on stage 0 to 7 of ACI was transformed to z-value. The graph of log size cut-off and Z-value gave a straight line. The MMAD was obtained from median of cumulative percentage where z-value is 0.

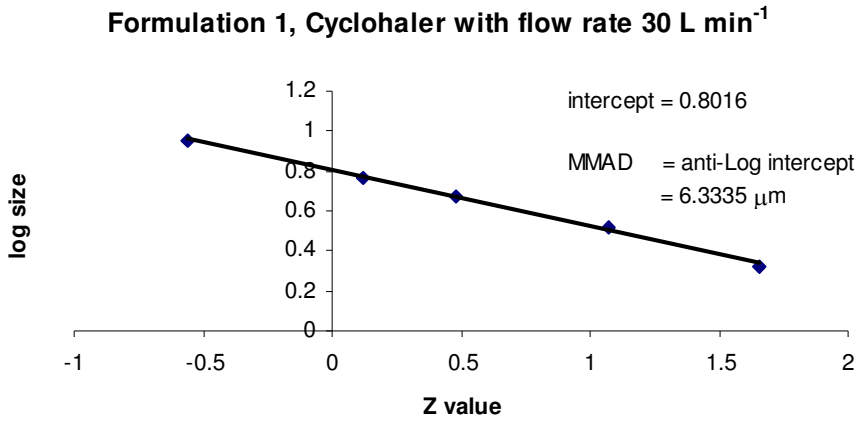


Figure 3.5 Determination of MMAD

3.5.2 Computer modeling of air-flow through pipes device

A key to understanding of the aerodynamic properties through pipe path is the Reynolds number (Equation 3.2), a dimensionless number that characterizes fluid flow through a pipe or around an obstacle such as an aerosol particle (Hinds, 1982). In fluid mechanics, the Reynolds number is the ratio of inertial forces to viscous forces and consequently it quantifies the relative importance of these two types of forces for given flow conditions. Thus, it is used to identify different flow regimes, such as laminar or turbulent flow.

$$Re = \frac{\rho V d}{\eta}$$

ρ = gas density (g/cm^3)

V = free-stream fluid velocity (cm/s)

d = characteristic distance or pipe diameter (cm)

η = gas viscosity ($dyn \cdot s/cm^2$)

Equation 3.2 Reynolds number equation

Laminar flow occurs at low Reynolds numbers, where viscous forces are dominant, and is characterized by smooth, constant fluid motion, while turbulent flow, on the other hand, occurs at high Reynolds numbers and is dominated by inertial forces, producing random eddies, vortices and other flow fluctuations.

In this study, the computational fluid dynamics software was used to compare fluid air flow properties between curved and straight pipe. At first, the Gambit 2.2.3 (Fluent Inc.) was used to draw the inner volume 3-D mapping of two type of pipes and then calculated mesh, inlet air-flow and outlet air-flow (Figure 3.6). The mesh file was exported from Gambit to solve in the three conservation types of mass, momentum, and energy equations under the steady laminar flow conditions by Fluent 6.2.16. The partial differential equations used in this study was the finite volume method, which divides space into regions or volumes and computes the change within each volume by considering the flux (flow rate) across the surfaces of the volume. Then, calculate pressure drop through devices, air velocity vector and cell Reynolds number. All of output data were obtained from iterate solves process at least 100 times.

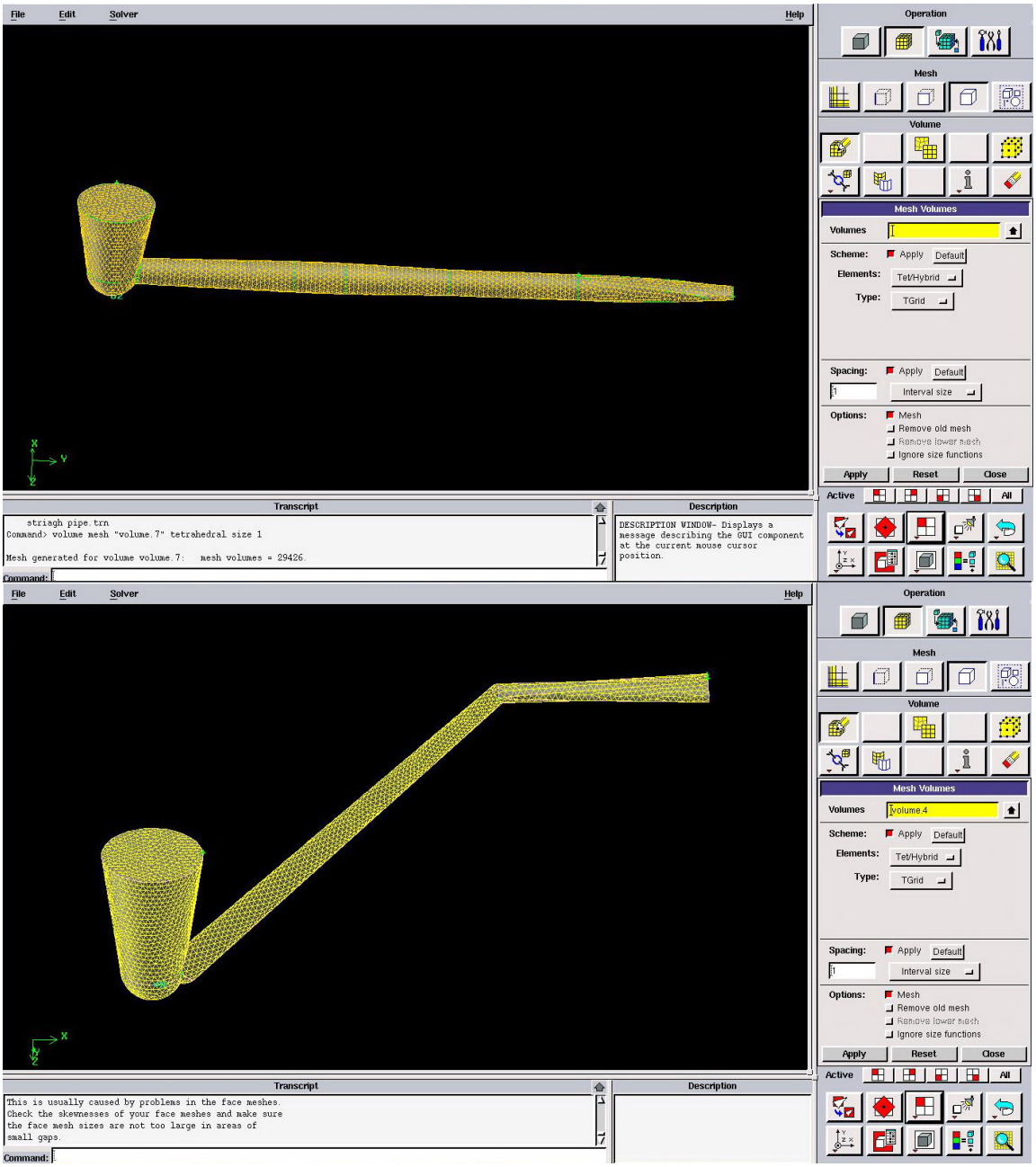


Figure 3.6 Calculated meshing pipes volume from Gambit 2.2.3