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An Efficient and Cost-effective Nose-only Inhalational Chamber for Rodents: Design, Optimization and Validation

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ABSTRACT

The mainstay treatment of pulmonary disorders lies around the direct drug targeting to the lungs using a nebulizer, metered-dose inhaler, or dry powder inhaler. Only few inhalers are available in the market that could be used for inhalational drug delivery in rodents. However, the available rodent inhalers invariably require high cost and maintenance, which limits their use at laboratory scale. The present work, therefore, was undertaken to develop a simple, reliable and cost-effective nose-only inhalation chamber with holding capacity of three mice at a time. The nebulized air passes directly and continuously from the central chamber to mouthpiece and maintains an aerosol cloud for rodents to inhale. Laser diffraction analysis indicated volume mean diameter of 4.02 ± 0.30 μm and the next-generation impactor studies, however, revealed mean mass aerodynamic diameter of 3.40 ± 0.27 μm, respectively. An amount of 2.05 ± 0.20 mg of voriconazole (VRC) was available for inhalation at each delivery port of the inhaler. *In vivo* studies indicated the deposition of 76.12 ± 19.50 μg of VRC in the mice lungs when nebulized for a period of 20 minutes. Overall, the developed nose-only inhalation chamber offers a reliable means of generating aerosols and successfully exposing mice to nebulization.

KEYWORDS: Nebulizer, Aerosols, Voriconazole, Preclinical, Spraytec.

INTRODUCTION

Inhalational drug delivery has been gaining immense importance in the treatment of various respiratory disorders for the last two decades. It offers distinct advantages of rapid onset of action, thin epithelial barrier, reduced dosage amount, localized action, avoidance of first-pass effect and gastrointestinal (GI) problems (1-3). A wide range of inhalational devices like nebulizers, dry powder inhalers (DPIs) and metered dose inhalers (MDIs) are available in the market to deliver drug molecules effectively to the human lungs (4-6). However, the availability of inhalation devices for carrying out the preclinical testing in rodents of drug therapeutics or their novel formulations at laboratory scale is quite limited.

26 Majorly, two types of inhalation exposure chambers, *viz.,* whole-body exposure and head/nose-
27 only exposure have been employed in the preclinical testing of inhalational drug products (7-
28 9). Other direct instillation methods, like intratracheal instillation or dry powder insufflation,
29 have also been used by the researchers to introduce the drug therapeutics directly to the lungs
30 (10, 11). Nevertheless, these commercially available rodent inhalers are too costly to meet up
31 the standard laboratory requirements, particularly for scientists working in developing
32 countries. To overcome such issues, some of the researchers have built their in-house inhalers
33 in order to conduct preclinical testing in rodents (12-15). The initial in-house models reported
34 in the literature consisted of glass bottles, where the rodents were placed either inside the bottles
35 (16) or in a side-exit of the bottle for inhalation (17). Tests were also conducted using inhalation
36 boxes, where cotton pieces or filter disks moistened with test material were placed and fixed
37 inside the box for inhalation by the rodents (18, 19). Some researchers have also used square-
38 shaped or cylindrical inhalation chambers, connected with nebulizers, for the generation of an
39 aerosol cloud for inhalation (20, 21).
40 Development and validation of a nose-only inhalation chamber for mice was reported by Kaur
41 *et al.* (2008) for delivering microparticles (MPs) of rifabutin in the form of dry powder (10).
42 Out of the 20 mg of fluidized MPs, about 2.5 mg were collected at the delivery port in 30 s of
43 operation, but only 61.5 µg of drug was inhaled by the mice. Albeit the design of the inhaler
44 was quite simple and easy to fabricate, yet it suffered from serious limitations of inconsistency
45 (being manually operated) and time-consumingness (being applicable for one animal at one
46 time). Subsequently, Yi *et al*. (2013) fabricated and validated a whole-body exposure chamber
47 to study the toxicity profile of titanium oxide nanoparticles (NPs) in rodents (22). The inhalation
48 chamber consisted of an aerosol generator, exposure chamber and a monitoring system. The
49 aerosols, generated from dry powder containing NPs were delivered to the exposure chamber
50 at a flow rate of 90 liters per minute. Despite the attainment of controlled and uniform aerosol

51 atmosphere of NPs during the whole process of inhalation exposure, several drawbacks were
52 associated with this type of inhalation chamber. These encompassed the use of a large amount
53 of formulation or drug being tested, aerosol exposure to other parts of rodent like skin or fur,
54 and acute requirement of excellent mixing for attaining uniform distribution of aerosol within
55 the chamber (1, 23). Sinha *et al*. (2013) also developed a nose-only inhalation chamber for dry
56 powder insufflation and nebulization taking six rodents at one time (13). The inhalation
57 chamber was made up of centrifuge tubes and a polypropylene-rectangular box, employing
58 voriconazole (VRC; in solution and micronized form) as a model drug. *In vivo* drug deposition
59 was found to be significantly higher in case of DPI (*i.e.,* 80–130 μg/g) vis-à-vis the nebulizer
60 (*i.e.,* 40-68 μg/g). The developed inhaler, however, had limitations of large inhalation chamber
61 and inability to keep the holding chambers at equidistant levels from the inlet (*i.e.,* DPI or
62 nebulizer), resulting eventually in uneven distribution of the inhalable dose. Recently, Silva *et*
63 *al.* (2017) developed and evaluated a round-wall glass inhalation chamber for preclinical testing
64 in rodents connected to a nebulizer on the upper side and animal holders the lower side (24).
65 Activation of the nebulizer passed air directly from the central portion to the holding chambers,
66 while the vapors within the chamber were collected and analyzed using gas chromatography.
67 Significant loss, however was noticeable, ostensibly owing its large central chamber (~8 cm
68 area), thus limiting its usage for the aerosolization of expensive drugs. Besides inhalation of
69 drugs, nose-only inhalation chambers have also been reported for the rodents for nebulizing
70 chronic wasting disease prions, infective bioaerosols and aqueous solutions of radiolabelled
71 human serum albumin formulation (12, 25, 26). Various pitfalls associated with these inhalation
72 chambers included complex and costly design set-up and difficulty to emulate the same at
73 laboratory levels.
74 The present research work, thus accordingly was undertaken to develop a simple, efficient,
75 reliable, reproducible and low-cost nose-only inhalation exposure system with an aim to

76 minimize the aerosol loss in the central chamber and to meet up the standard requirements of
77 preclinical testing at the small-scale laboratory. The developed inhalation chamber was
78 evaluated and validated using *in vitro* and *in vivo* tests both, employing VRC as the model drug.

79 **MATERIALS AND METHODS**

80 **Instrument and Reagents**

81 The jet nebulizer was procured from Philips, New Delhi, India. Centrifuge tubes (50 mL) were
82 purchased from Tarsons, New Delhi, India. VRC was generously supplied as a gift sample by
83 M/s Panacea Biotech, New Delhi, India. The HPLC-grade solvents, *viz.,* acetonitrile (ACN)
84 and methanol, were purchased from M/s Fisher, New Delhi, India. All other chemicals used
85 were of high purity or analytical grade and were employed as such obtained.

86 **Design of Inhalation Chamber**

87 Based on the know-how gained from the previous inhaler reports, the nose-only inhalation
88 chamber was designed using readily available objects. The low-cost inhalation chamber
89 consisted of five major parts, a) nebulizer as aerosol generator; b) a three-way splitter as central
90 chamber; c) flexible tubing as a linker, *i.e.,* connect nebulizer to three-way splitter; d) a small
91 rectangular vial (5.5 cm length, 3 cm diameter) as mouthpiece for rodents and e) centrifuge
92 tubes (50 mL) as mice holding chambers or restrainers.

93 **Analytical and Bioanalytical Method Development**

94 Analysis of VRC was performed as per the analytical method developed and validated in our
95 laboratory using a mobile phase consisting of ACN and acetic acid solution (50:50), at a flow
96 rate of 1 mL/min. The entire analysis was conducted employing a reversed-phase C_{18} column,
97 250 x 4.6 (mm) with a particle size of 5 µm (Purospher® STAR, Merck) and a PDA detector
98 using a wavelength of 256 nm under isocratic conditions.

99 A standard stock solution of VRC (10 mg/mL) was prepared in methanol: water (1:1), which
100 was further diluted to obtain a drug concentration of 100 µg/mL. Various working standard

101 solutions *i.e.,* 0.1-50 µg/mL were serially prepared from the stock solution (100 µg/mL). For
102 bioanalytical estimation, an aliquot of 200 µL of lung homogenate was added to 200 µL of
103 working standard solutions with volume make up to 1 mL using ACN. The mixture blend was
104 vortexed, centrifuged and filtered through a 0.22 µm syringe filter prior to subjecting for HPLC
105 analysis.

106 **Determination of Microdroplet Size of an Aerosol**

107 The microdroplet size of an aerosol was measured using the Spraytec laser diffraction
108 instrument (Malvern Instruments, UK), equipped with a He-Ne laser. A lens with a focal length
109 of 300 mm was used covering microdroplet size between 0.1 and 900 µm. Briefly, 5 mL of the
110 sample was loaded on to the jet nebulizer and placed perpendicularly to the laser lens line of
111 the instrument at a distance of 3 cm from the laser beam (27). The values of volume mean
112 diameter (VMD), geometric standard deviation (GSD) and the fine particle fraction (FPF) were
113 computed using the Spraytec version 3.20 software after passing aerosol droplets through the
114 laser beam (28).

115 **Determination of Pulmonary Deposition by Next-Generation Impactor**

116 Pulmonary deposition studies were performed using a Next-Generation Impactor (NGI) at a
117 flow rate of 15 L/minute (29). The nebulizer was connected to the induction port of NGI
118 (COPLEY Scientific, UK) with a mouthpiece adapter, filled with 5 mL of inhalation solution
119 with an aerosol collection time of 5 minutes. Once the measurement was completed, the cup
120 holder tray was removed and each of its stages was washed with 10 mL of extraction solvent
121 (*i.e.,* methanol), filtered and subjected to HPLC analysis for determining the VRC content at
122 each stage of the impactor. The mass median aerodynamic diameter (MMAD) and geometric
123 standard deviation (GSD) were calculated using online MMAD calculation software (30).
124 Emitted dose (ED), *i.e.,* the total amount of drug emitted from the inhaler device and fine

125 particle fraction (FPF), *i.e.,* the ratio of the mass of particles less than 5 μm to the emitted dose,
126 was also calculated (31).

127 **Determination of Nebulized Air-Flow at Each Delivery Port**

128 The nebulized airflow reaching each delivery port was determined with the help of a gas flow
129 meter MGF 505 (Metrex, New Delhi, India). The flow meter was attached to one of the three
130 outlets of the central chamber, with the rest of the two outlets closed with the help of a glass
131 stopper. The airflow value of all the three delivery ports was determined in order to confirm the
132 uniformity of airflow during operation (24).

133 **Determination of Dose Available for Inhalation**

134 The initial characterization of the exposure chamber was performed using pre-weighed cotton
135 balls (250.12 ± 13.09 mg), placed close to the delivery port of the holding chambers (10).
136 Different concentrations of VRC (1-3 mg/mL) in a solution of normal saline were nebulized for
137 a period of 20 minutes. During operation, the surface of the cotton ball was exposed to the
138 aerosol generated from the nebulizer. After corresponding periodic time-intervals, the nebulizer
139 was turned-off, the balls were removed using forceps from the tube and were weighed on a
140 precision analytical balance (Mettler Toledo ME204, Ohio, USA). Furthermore, VRC was
141 estimated quantitatively employing an HPLC-based analytical technique after soaking the
142 cotton ball in a solvent blend of methanol and water (1:1) for 2 h, followed by bath sonication
143 for 10 minutes and filtration using 0.22 μm filters. Once the amount of the VRC at each delivery
144 port was confirmed, the performance of the developed inhalation chamber was further tested in
145 the animals studied.

146 **Validation of Inhaled Voriconazole in Balb/c Mice**

147 The animal experiments were carried out after obtaining the requisite ethical approval from the
148 Institutional Animal Ethics Committee of Panjab University, Chandigarh, India
149 (PU/45/99/CPSEA/IAEC/2019/243). Balb/c mice weighing 23 ± 2 g, were procured and

150 provided with a standard diet and water *ad libitum.* Prior to each experiment, mice were fasted
151 for 12 h with free access to water. Animals received nebulization of 3 mg/mL of VRC (Vorier,
152 Aspiro Pharma, Telangana, India) for a period of 20 minutes. During the experiment, the test
153 solution was aerosolized using a jet nebulizer, *i.e.,* Philips Respironics InnoSpire compressor
154 nebulizer with a volume capacity of 15 mL. Ten milliliters of VRC solution in normal saline (3
155 mg/mL) was placed into the nebulizer fluid cup, mice were loaded into the apparatus and the
156 entire chamber was placed in a secondary enclosure in a well-vented room. The aerosol
157 generated by the nebulizer entered the central chamber and reached the delivery port. Each
158 animal was positioned in such a way that the nose of the mice was exposed to the delivery port.
159 The air exhaled by the animal escaped from the mouthpiece *via* a small opening present at the
160 upper wall near the apex. As the central chamber and mouthpiece were airtight, no air entered
161 or left the exposure system except *via* the aerosol delivery and exhaust. At specified time
162 intervals of 0.5, 2 and 6 h, animals (n=3) were sacrificed by cervical dislocation, the lungs were
163 removed, homogenized in PBS 7.4 (5 mL) for 5 minutes employing a tissue homogenizer
164 (Heidolph, RZR 2011, Germany) and stored at -20°C until analyzed. At the time of experiment,
165 the samples were thawed and 200 µL of lung homogenate was added to 1 mL of ACN followed
166 by vortexing for 20 minutes. The samples were centrifuged for 10 minutes at 10,000 rpm (9055
167 × g) and the supernatant was then filtered through 0.22 µm syringe filters prior to HPLC
168 analysis. The percent drug deposition in lungs *in vivo* was measured by dividing the total VRC
169 deposited in the lung tissue with the amount of drug available for inhalation at the delivery
170 ports, obtained during the *in vitro* studies.

171 **Statistical Analysis**

172 Values were listed as means ± SD. Statistical comparison of means was performed by unpaired
173 Students t-test and one-way ANOVA. Statistical analysis was performed using GraphPad
174 Prism, Version 4.03.

175 **RESULTS AND DISCUSSIONS**

176 **Design of Inhalation Chamber**

177 The development of a low-cost nose-only inhalation chamber started with a review of various
178 inhaler devices reported in the literature to date (12, 14, 25, 26, 33-35). After analyzing the data,
179 a final sketch of the model equipment was prepared with an aim to minimize the aerosol loss in
180 the central chamber (*i.e.,* connect nebulizer to animal restrainer), preferably using readily
181 available, inexpensive, easy to disassemble and clean objects.

182 In order to design the apparatus for use by three animals at a time and to minimize the drug
183 wastage, a three-way splitter (Fig. **1**) of diameter 1 cm, approximately 8-fold less than the
184 recently developed inhaler (24), was used as the central chamber. The trifurcations of the central
185 chamber were placed at the equidistant levels from the inlet to ensure uniformity in the dose
186 delivered to each mouthpiece and ultimately to the delivery ports of the animal restrainers.
187 Moreover, the small size of the central chamber reduced the time required for its saturation,
188 thus economizing both time as well as drug amount. A small rectangular vial was used as a
189 mouthpiece that was connected to each outlet of the central chamber. A small hole or vent was
190 also made in the wall of each mouthpiece, near the rim of the screw cap, in order to minimize
191 any pressure build-up inside the aerosol chamber.

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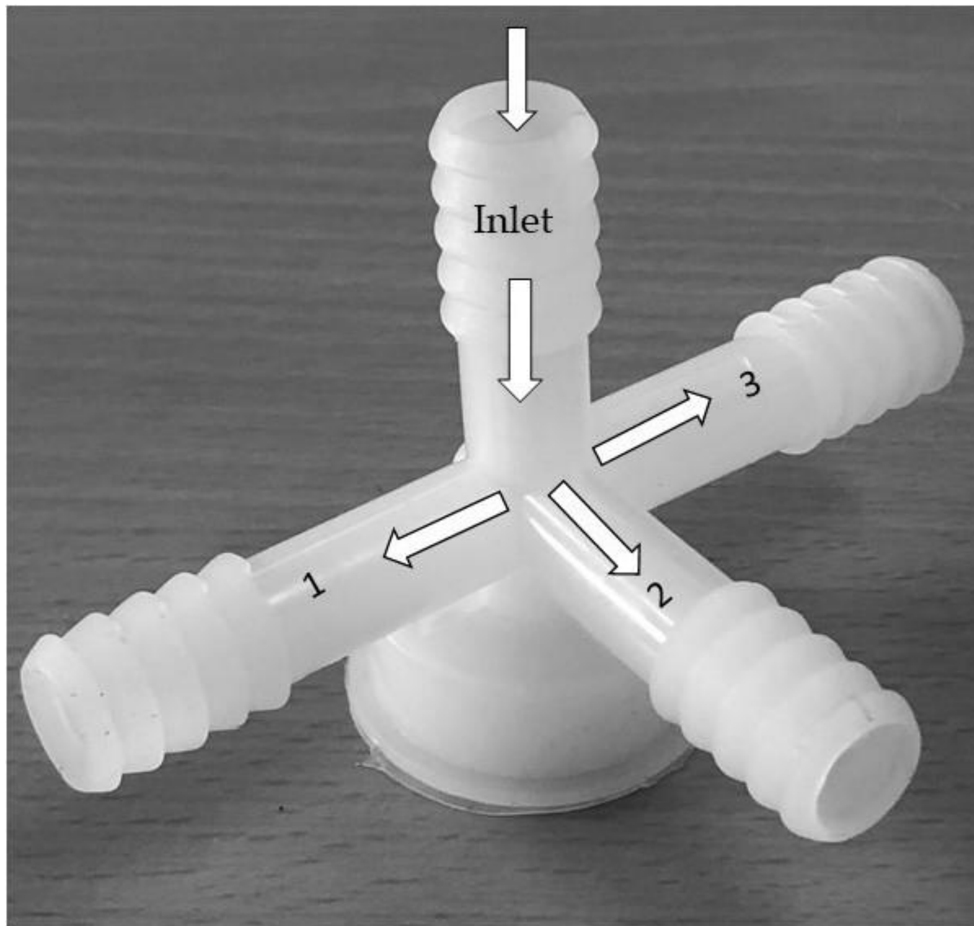


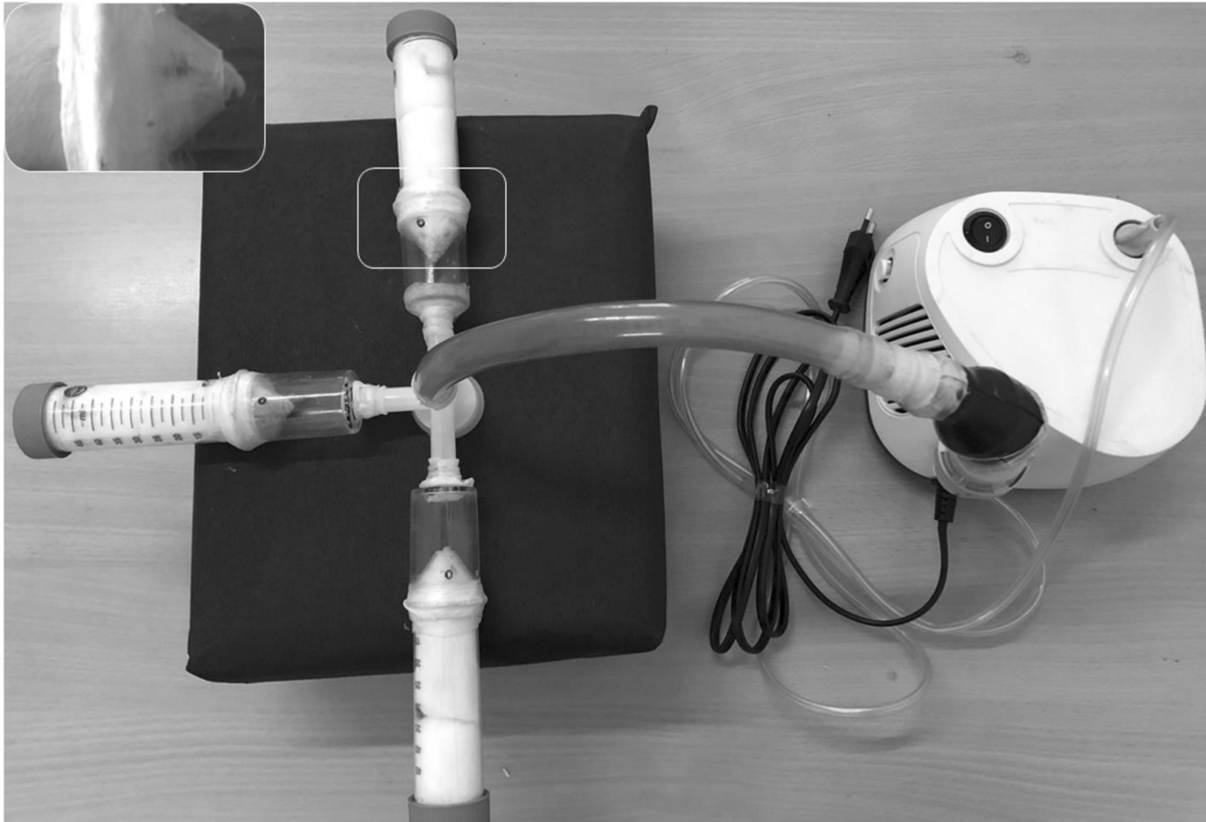
193 Fig. **1**. A three-way splitter as central chamber depicting the flow pattern of the aerosol.

194 Centrifuge tubes with a diameter of 1.8 cm were used as the mouse-holding chambers or
195 restrainers, as these are routinely used in the laboratories and are small enough to prevent any
196 plausible change in the direction of movement by the animals. Moreover, their cylindrical
197 structure with a narrow front and large aperture at the distal part allowed easier introduction
198 and removal of an animal weighing 23 ± 2 g. The tips of the centrifuge tubes were removed to
199 make a hole of around 0.9 cm diameter, so that nose of the mouse can easily be inserted into
200 these. Care was exercised to leave a smooth edge to avoid any discomfort to the animal. The
201 holding chambers were designed in such a way that mainly the nose of the mouse was exposed
202 to the aerosol cloud, as shown in the photograph (Fig. **2).** The animals were restrained with the
203 help of cotton within the animal holders.

204

205 Fig. **2**. Aerosol-exposure to mice in the holding chamber.

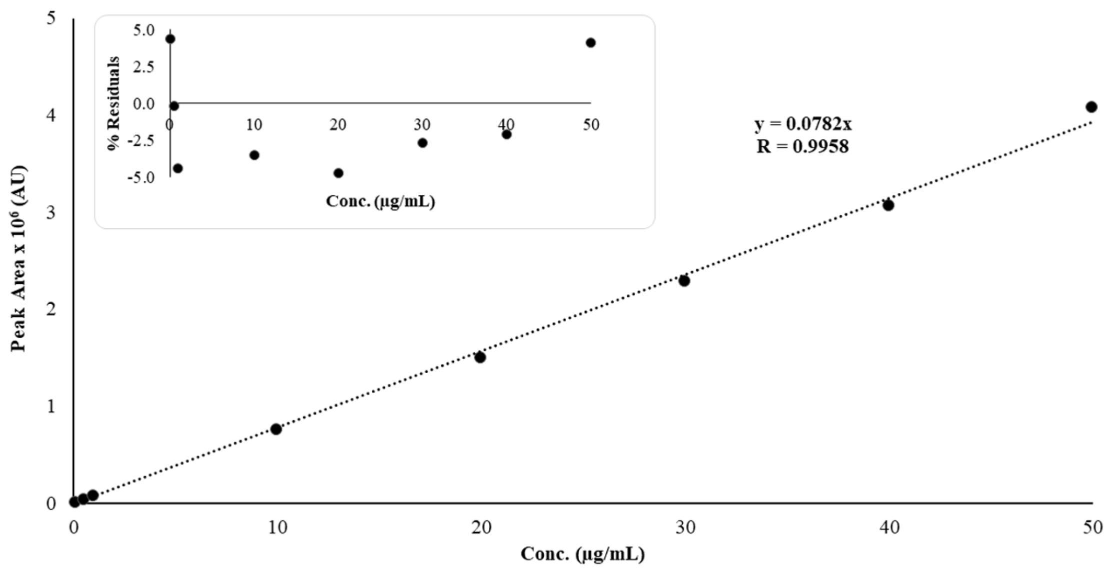
206 A flexible tubing, with 25 cm length and 1.2 cm diameter, was employed to connect the mouth
207 of the nebulizer with the central portion of the three-way splitter. It serves the purpose of
208 transferring the mist generated from the nebulizer to the delivery port through the central
209 chamber. The fabrication of the inhalation chamber is depicted in the photograph (Fig. **3**). The
210 compressor attached to the nebulizer provides a positive pressure of 12.1 ± 1.2 psi for the
211 generation of an aerosol mist. After the final assemblage, the inhalation chamber was checked
212 for any leakage by nebulizing it with distilled water. Any leaks detected were sorted and fixed
213 until the instrument was flawlessly leak-proof.

214 

215 Fig. **3.** Design of the inhalation chamber coupled with a nebulizer.

216 **Analytical and Bioanalytical Method Development**

217 Linear calibration curves were generated with working standards for the quantification of VRC
218 in cotton balls and in the lungs. Validation of linearity range was carried out by constructing
219 the residuals plots (inset), which indicated the percent deviation in responses, well within ± 5%
220 limit of the working VRC concentrations as illustrated in Fig. **4 (A, B).** A chromatogram
221 showing the peak of VRC in methanol: water and in lung homogenate is illustrated in Fig. **5**
222 **(A, B).**



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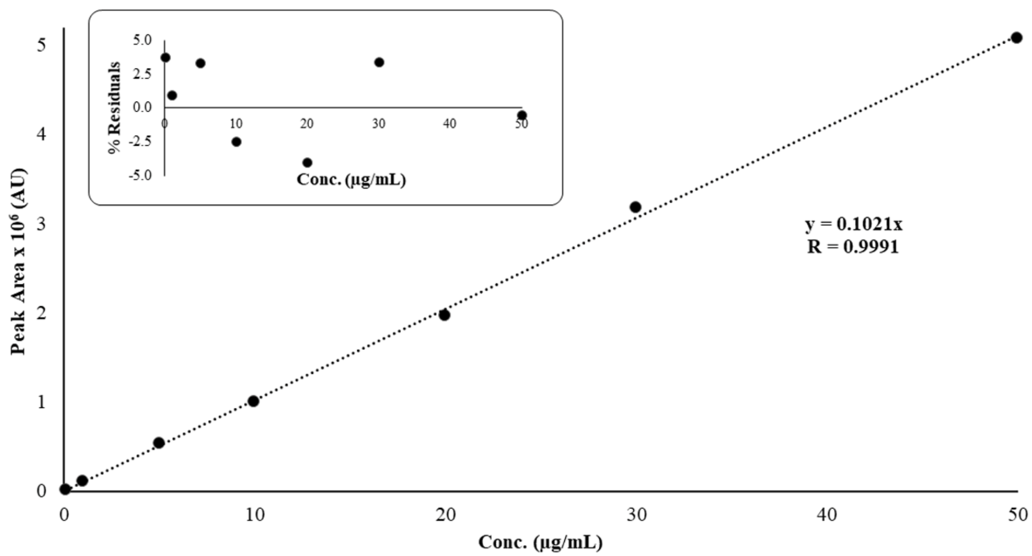

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225 Fig. **4:** Calibration plot of VRC in A) methanol: water and B) lung homogenates. The inset
226 depicts the corresponding residual curves.

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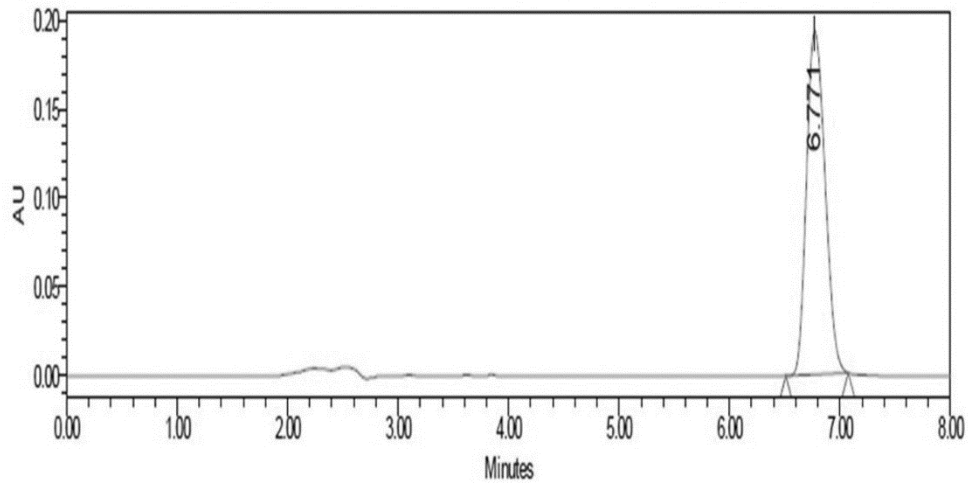

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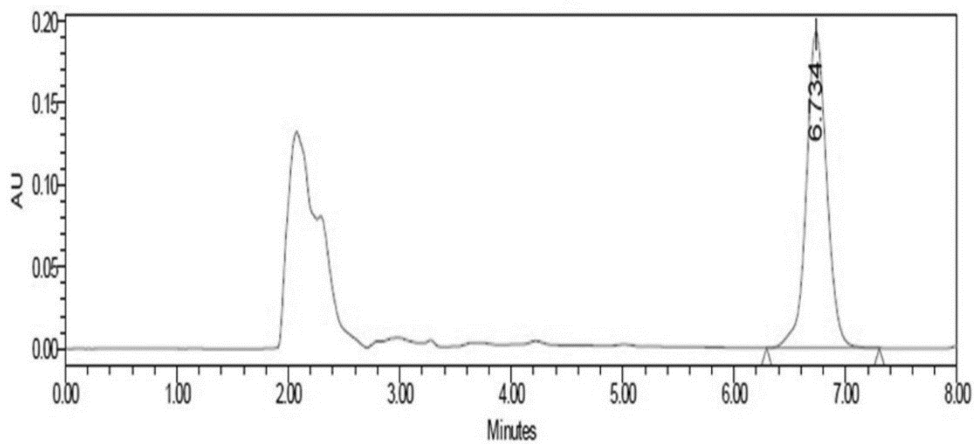

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230 Fig. **5**. The chromatogram of voriconazole in (A) methanol: water (B) lung homogenates.

231 **Determination of Microdroplet Size of Aerosol**

232 Fig. **6** represents the frequency and cumulative volume distribution profile of the aerosol
233 generated through a jet nebulizer. Laser diffraction analysis yielded a VMD of 4.02 ± 0.30 µm
234 and GSD of 1.79 ± 0.02 µm. The proportion of microdroplets with a diameter below 5 µm, *i.e.,*
235 the fine-particle fraction (FPF), was found to be 62.62 ± 0.45 %. Thus, VMD of less than 5µm
236 depicts the potential of the generated aerosol to deposit in the lungs.

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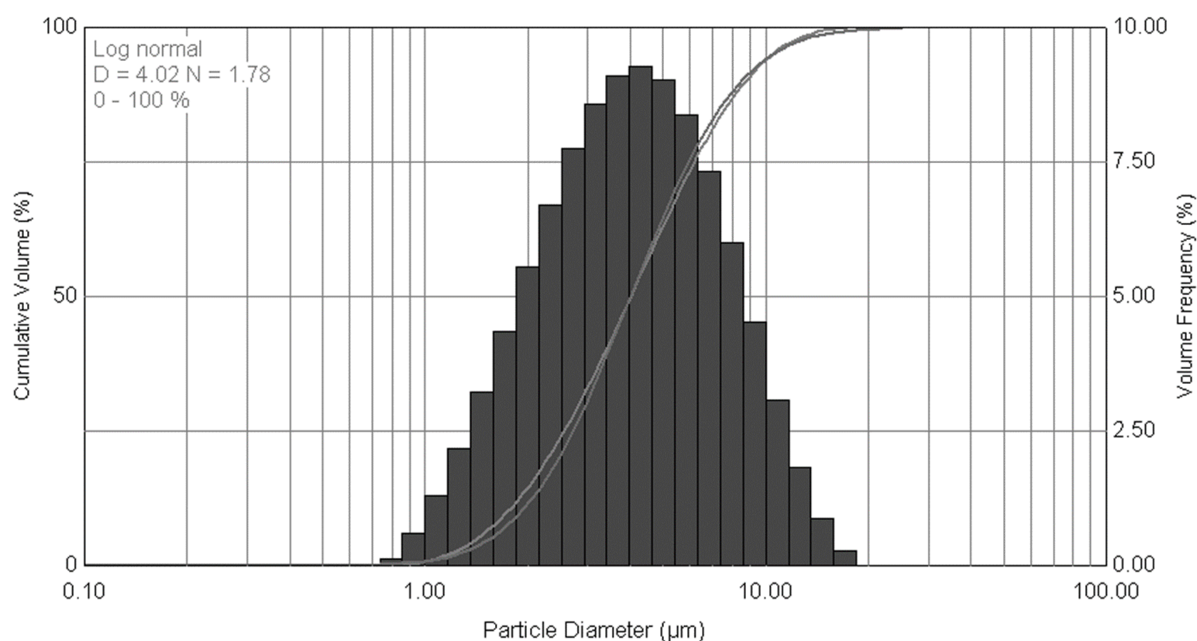

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242 Fig. **6:** Particle size distribution of voriconazole microdroplets.

243 **Particle Deposition Studies by Next-Generation Impactor**

244 Fig. **7** illustrates the deposition of VRC at various stages of the impactor, indicating maximum
245 drug deposition on Stage 3 (*i.e.,* cutoff of 5.4 µm), Stage 4 (*i.e.,* cutoff of 3.3 µm) and Stage 5
246 (*i.e.,* cutoff of 2.08 µm) of the impactor. The MMAD and GSD were found to be 3.40 ± 0.27
247 µm and 2.14 ± 0.10 µm, respectively, with 49.30 ± 3.66 % of dose emitted from the nebulizer
248 and 40.00 ± 2.47 % of FPF. The observed MMAD was found to be less 4 µm, construing that
249 the nebulization of model drug exhibits targeting potential in the airways, primarily by the
250 mechanism of sedimentation and diffusion (1, 36). In this context, a GSD of >1.2 known to
251 indicate a heterodisperse aerosol size distribution, in accordance with the results generally
252 observed with most of the other aerosols (37-39).

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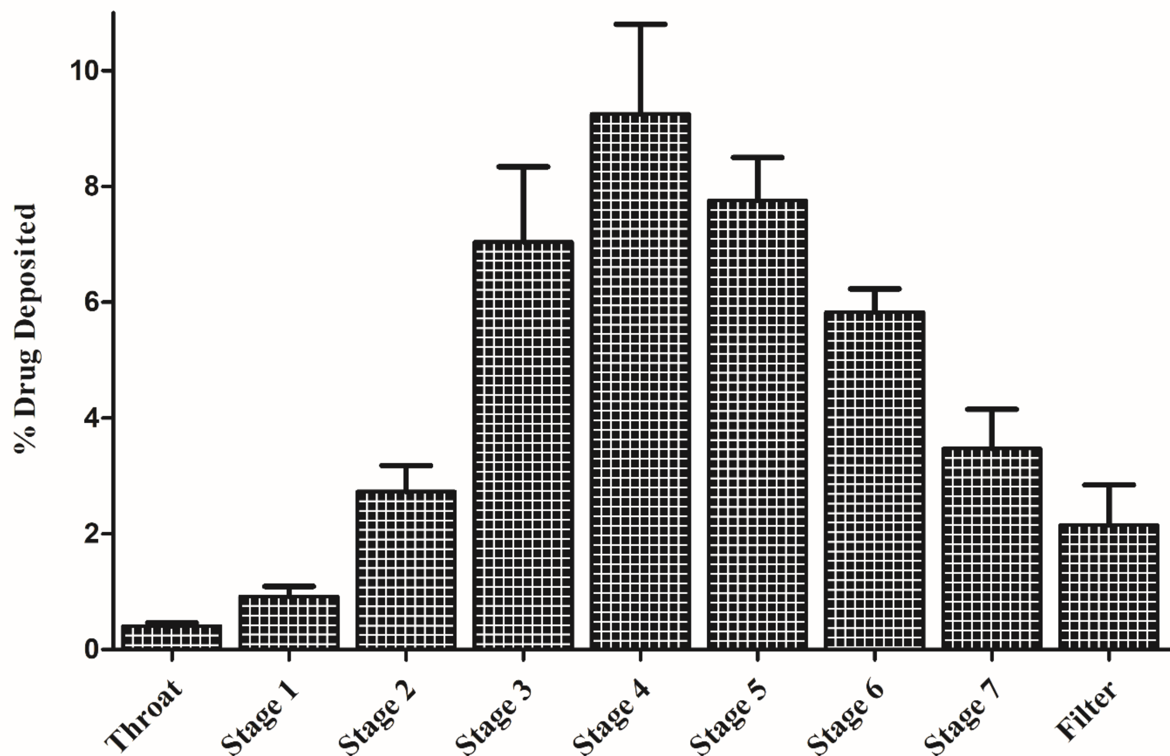


254 Fig. **7**: *In vitro* pulmonary deposition studies on percentage of voriconazole deposition using a
255 next generation impactor at 15 L min^{-1}. Each crossbar indicates 1 SD.

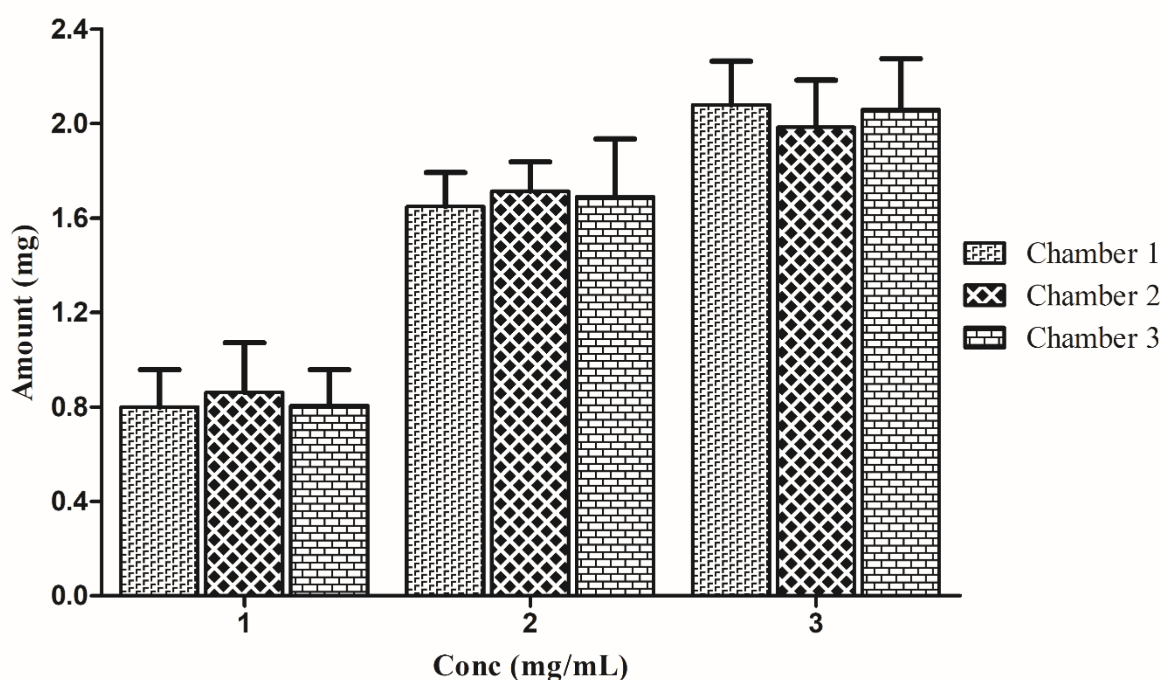
256 **Determination of Air-flow at each Delivery Port**

257 The airflow observed at each delivery port was found to be quite consistent, *i.e.,* close to 4.21
258 ± 0.13 L/min at each outlet, when the other two outlets were closed. This could be attributed to
259 the uniformity maintained by the three-way splitter in delivering the aerosolized drug to each
260 delivery port of the animal holding chamber. Further, the air reaching at all the delivery ports
261 could be analyzed by dividing the above value by number of delivery ports (*i.e.,* 3) which is
262 1.40 ± 0.13 L/min at each delivery port.

263 ***In vitro* Optimisation**

264 Before the start of the experiment, the nebulizer was primed for 1 minute to saturate the central
265 chamber and to maintain a steady flow to the delivery port. The *in vitro* test conducted with
266 cotton balls revealed the enhancement in the weight of cotton balls by small increments of 5.42
267 ± 0.18 mg. A total of 10 mL solution was nebulized initially, out of which 5.17 ± 0.25 mL was

268 remaining after nebulization of 20 minutes. An amount of 2.05 ± 0.20 mg (14.5 %) of VRC was
269 recovered at the highest concentration tested (3 mg/mL) at each of the delivery ports with a
270 coefficient of variation less than 18%. In total, 6.12 ± 0.60 mg drug (43.4%) was available at
271 all of the delivery ports with 14.10 ± 0.15 mg of total drug nebulized and 15.90 ± 0.75 mg of
272 total amount corresponds to dead dose in the nebulizer. The dose recovered has been 10-folds
273 and 1.2-folds higher than the previously reported articles (10, 14). Fig. **8** presents the VRC
274 fraction obtained across each delivery port, connected to a central chamber through the
275 mouthpiece. As observed, the fraction of VRC recovered increases in the holding chambers, as
276 the concentration approaches towards maximum. Moreover, it was noted that the relative mass
277 fraction did not vary substantially among the three-exposure ports at each of the concentration
278 tested ($p>0.05$), evidently owing to the equidistant position of all the holding chambers with
279 respect to the central chamber.



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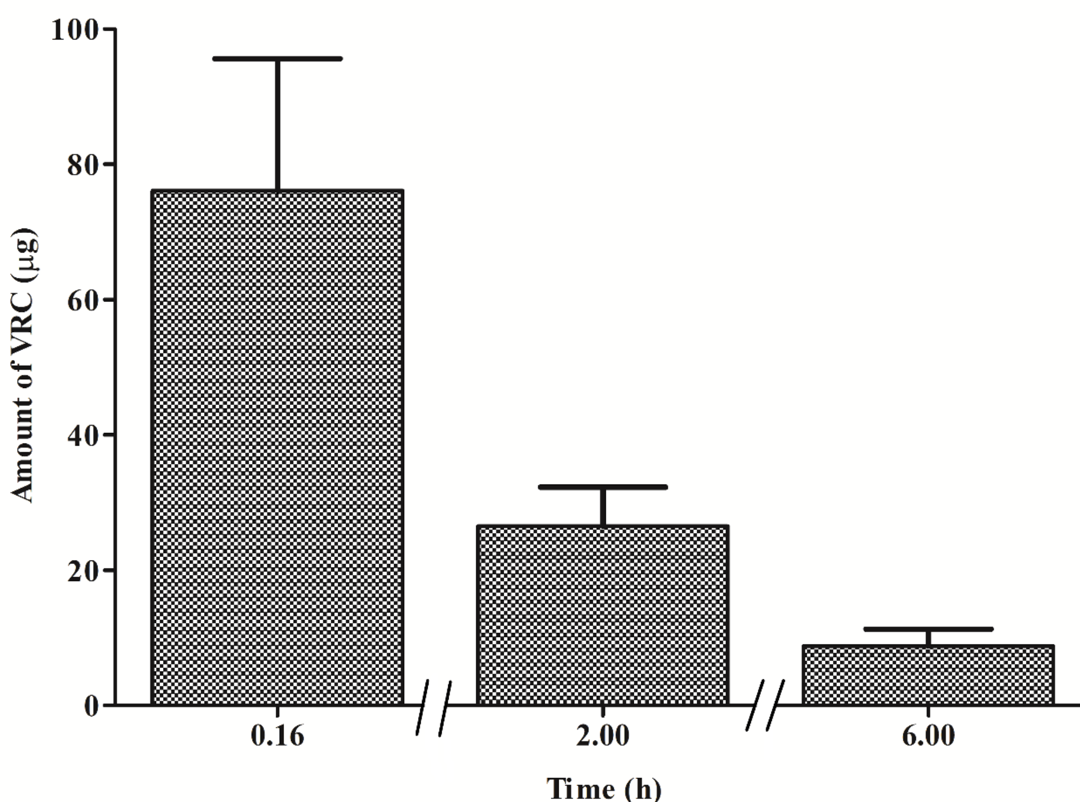
281 Fig. **8**: Optimisation of dose available at each of the delivery ports at varied concentrations of
282 voriconazole. Each crossbar indicates 1 SD.

283

284 **Validation of Inhaled Voriconazole in Balb/c Mice**

285 Fig. **9** illustrates the biodistribution profile of VRC in the lungs following inhalation using a
286 nebulizer. An amount of 76.12 ± 19.50 μg (3.81 ± 0.97 %) of VRC was deposited in the whole
287 lungs following nebulization for 20 minutes (Table 1), followed by a declining trend up to 6 h.
288 This could be attributed to the fast rate of drug diffusion of the previously solubilized VRC
289 molecule, large alveolar surface area and thin physiological membrane in the lungs. Therefore,
290 the results substantiate the successful application of the developed inhaler device in the
291 nebulization of VRC microdroplets in mice lungs.



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293 Fig. **9:** Lung distribution profile of voriconazole in mice lungs after nebulization for
294 20 minutes. Each crossbar indicates 1 SD.

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296

297 **Table I:** Data pertaining to the emitted and inhaled drug dose.

298

Parameter(s)	Mean ± SD
Initial dose in nebulizer (mg)	30.00 ± 0.12
Emitted dose (mg)	14.10 ± 0.15
Dose available at each delivery port (mg)	2.05 ± 0.20
Percent of drug available for inhalation (%)	14.53 ± 1.28
Dose deposited in mice lungs (µg)	76.12 ± 19.50
Percent *in vivo* deposited (%)	3.80 ± 0.97

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305 **CONCLUSIONS**

306 The present study demonstrates the development of a simple, cheap and efficient nose-only
307 inhalational chamber for routine analysis of the aerosols, generated through nebulization for
308 use in rodents (20-25 g). Reliability of the inhalation chamber was validated, attributable to the
309 uniform distribution ($p > 0.05$) of the test particles across the three-delivery ports. Moreover,
310 reduction in the area of the central chamber reduces drug wastage, thus economizing the cost
311 for highly expensive drugs like VRC and anticancer drugs. Further, the apparatus could prove
312 to be a promising tool for preclinical testing of drugs *per se* or their novel formulations,
313 administered through nebulization at the laboratory scale. Moreover, the device can also be
314 opted for short-or long-term toxicological investigations in rodents. The benefits of the device
315 can also be subsequently extended for usage in larger rodents (around 250g) by just switching
316 over to animal restrainers.

317 **CONFLICT(S) OF INTEREST**

318 The authors confirm no conflict of interest.

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