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**Citation:** Mashat M, Clark BJ, Assi KH et al (2015) In vitro Performance Assessment of Recent Nebuliser Delivery Systems for Nebulisation of Approved Aerosolised Tobramycin (TOBI)®. Journal of Applied Biopharmaceutics and Pharmacokinetics. 3: 34-46.

# *In vitro* Performance Assessment of Recent Nebuliser Delivery Systems for Nebulisation of Approved Aerosolised Tobramycin (TOBI)®

Marwan H. Mashat[*,1], B.J. Clark[2], K.H. Assi[2] and H. Chystyn[3]

[1]*Makkah Toxicology Centre, P.O. Box 4123, Makkah, Saudi Arabia*

[2]*School of Pharmacy and Institute of Pharmaceutical Innovation, University of Bradford, Bradford, UK*

[3]*School of Applied Sciences, University of Huddersfield, Huddersfield, UK*

**Abstract:** TOBI® is a recently marketed preservative and sulphate free tobramycin formulation approved by FDA for maintenance therapy for patient with cystic fibrosis. The performance of selected recent nebuliser delivery systems has been assessed using the developed method to determine the optimum combinations to deliver approved tobramycin inhaled solution (TOBI)®. A simple, sensitive and specific high performance liquid chromatographic method has been developed and used to quantitative determination of the aminoglycoside tobramycin following pre-column derivatisation with phenylisocyanate (PIC). The reaction time was 10 min at 80° C and the resulting derivative was stable for five days at room temperature. The quantitative performance of the assay was further improved by using another aminoglycoside (neomycin) as internal standard. The stable resulting PIC-tobramycin derivative was separated using a HPLC 5μm Columbus C18 column (150x4.60 mm i.d, Phenomenex). The mobile phase was consisted of acetonitrile-glacial acetic acid-water (450:5:545, v/v/v) and ultraviolet detection at (240 nm). The proposed method showed good validation data. The standard curve was linear (n=5) at seven different concentrations, ranging from 20 to 140μg/ml and the correlation coefficient ($R^2$) of the regression line was 0.9995. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.86μg/ml and 2.62μg/ml, respectively. The relative standard deviation (RSD %) was less than 0.6% for intra-day assay (n=5) and 2.5% for inter-day assay (n=5). A number of nebuliser performance comparison studies have been demonstrated for aerosolise TOBI® to choice the optimum combination produces high repirable inhaled mass of tobramycin. The objective of this study was to evaluate the performance of recent nebuliser delivery systems to nebulise approved tobramycin inhaled solution (TOBI)®.

**Keywords:** Tobramycin, TOBI®, PIC derivatisation, HPLC, *In vitro* performance assessment, Nebuliser delivery systems.

## 1. INTRODUCTION

Aminoglycosides are a group of bactericidal drugs sharing chemical, antimicrobial, pharmacological, and toxic characteristics.

Tobramycin is a broad spectrum aminoglycoside antibiotic first isolated in 1957 by investigators of Eli Lilly Research Laboratories. Seven antibiotic factors were isolated from Streptomyces Tenebrarius [1], and factor 6 was subsequently designated as tobramycin [2]. Tobramycin exhibits a broad spectrum against aerobic gram-negative bacteria especially Pseudomonas and indole positive Proteus [3] that makes it the antibiotic of choice in the treatment of pulmonary infections.

Like the other aminoglycosides, tobramycin has narrow therapeutic range with potential nephrotoxicity and ototoxicity. Therefore, careful monitoring of the serum concentration of tobramycin is required to achieve the optimal therapeutic level and to minimise toxicity. Tobramycin is a highly polar base that exhibits poor oral bioavailability, low protein binding (10% of absorbed drug), and excreted unchanged. Furthermore, it has a polycationic nature and therefore, it cross membranes very poorly, and does not cross the blood brain barrier, the CNS, nor penetrate ocular tissues. Therefore, it is administered by injection for systemic infections or topically (direct to the site of infection) for topical infections. The systemic availability of tobramycin that reflects the mass of inhaled drug deposited in the lungs can be calculated from the urinary excretion.

TOBI® is specifically formulated for administration by inhalation. When inhaled, tobramycin is concentrated in the airways. The bioavailability of TOBI® may vary because of individual differences in nebuliser performance and airway pathology [4]. Following administration of TOBI®, tobramycin remains concentrated primarily in the airways.

The most important characteristic for nebuliser performance assessment is the respirable mass output and the aerosol droplets size that are produced by

*Address correspondence to this author at the Makkah Toxicology Centre, P.O. Box 4123, Makkah, Saudi Arabia; Tel: 00966 555503757; Fax: 00966 124321519; E-mail: mhalmashat@gmail.com

nebuliser device. The nebulisation time, simplicity of use, cost, and cleaning and sterilization requirements are also important characteristic for nebuliser performance assessment.

By using breathing simulator (Pari Respiratory Equipment, Inc) and eight stages Marple 298 Cascade Impactors (Copley Scientific Limited, UK). In this study, *in vitro* performance assessment of recent nebuliser delivery systems for nebulisation of approved aerosolised tobramycin (TOBI)[®].

This study include a simple and sensitive HPLC method following pre-column derivatisation with phenylisocyanate (PIC) for the determination of tobramycin in pharmaceutical formulations has been developed. Aminoglycosides and most amines have very low absorbance in the UV-region, because these drugs do not have a significant UV absorbance chromophore, and fluorescence [5]. Moreover, these compounds difficult to retain on conventional HPLC columns because of their highly polar polycationic nature. Therefore, derivatisation with a suitable absorbance enhancing or fluorescence-producing agent is necessary to increase the detectability of these compounds by producing a highly detectable product easily for detection. Recently, a validated method for the determination of tobramycin by capillary electrophoresis after derivatisation with OPA/mercapto-propionic acid (MPA) has been described [6].

A number of HPLC pre-column derivatisation methods have been demonstrated with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [7,18], 1-fluro-2,4-dinitrobenzene (FDNB) [4-7,26], O-phthaladehyde (OPA) [9-12] and 1-naphthyl isothiocyanate (NITC)[13]. The major drawback and limitation use of TNBS and FDNB reagents is due to their high toxicity and the main disadvantage of NITC reagent is length of time. Post-column derivatization methods have been demonstrated with o-phthaladehyde (OPA) [14-16]. The major disadvantage of the OPA reagent is the poor stability of the derivative.

Recently, two new methods have been reported with detection modes are not readily available in every laboratory; ion-pair liquid chromatography with pulsed electrochemical detection (LC-PED) [17] and liquid chromatography-tandem mass spectrometry (LC-MS-MS) [18] have been developed to determination of tobramycin without derivatisation with good repeatability.

The main advantages of the proposed method are its simplicity, sensitivity, accuracy and that it gives a high stable PIC-tobramycin derivative in a short analysis time.

## 2. EXPERIMENTAL

### 2.1. Materials and Chemicals

Tobramycin (98% purity) and neomycin sulphate (internal standard) were purchased from (Sigma-Aldrich, Louis, USA). phenylisocyanate (PIC) that used as derivatising reagent was obtained from (Fluka chemie Gmbh, Switzerland),Glacial acetic acid was supplied by BDH (Pool, UK). All solvents used; acetonitrile, and methanol were HPLC grade and purchased from Fisher Scientific UK Limited (Loughborough, UK). All solutions were prepared with ultra-pure milli-Q water further purified for HPLC by using a (Milli-Q water Millipore purification system, USA). TOBI[®] (300mg/5 ml, Chiron Corporation, Emeryville, USA). The Pari LC Plus[®] attached to Pari Boy*N*[®] compressor (Pari Respiratory Equipment, Inc). Sidestream[®] attached to Porta-Neb[®] compressor (Profiles, UK). Omron NE-U22[®] high and low frequency (Omron, Japan). Aeroneb Go[®] (Aerogen, Inc, USA). Breathing simulator (Pari Respiratory Equipment, Inc). Eight stages Marple 298 Cascade Impactors (Copley Scientific Limited, UK). Vacuum controller model Erweka TPK (Copley Scientific Limited, UK). Mass flow meter model PR400 (MKS Instruments, Andover, MA, USA).

## 3. METHODS

### 3.1. HPLC Method Development

#### 3.1.1. Chromatographic Conditions

The HPLC system consisted of Hewlett-Packard GmbH (HP) 1050 pump and autosampler connected to an on-line membrane degasser (Thermo Separation Product, California, USA). Hewlett-Packard (HP) 1050 series variable wavelength UV detector was set at wavelength (240 nm), linked to Prime multichannel data station software (HPLC Technology, Ltd, Herts., UK).

The chromatographic separation was carried out on a HPLC 5µm Columbus C18 column (150x4.60 mm i.d, Phenomenex, Macclesfield, UK) protected by a Phenomenex Security Guard cartridge column (C18, 4x3 mm i.d). The mobile phase consisted of acetonitrile-glacial acetic acid-water (430:5:565, v/v/v). The mobile phase was filtered and degassed through a
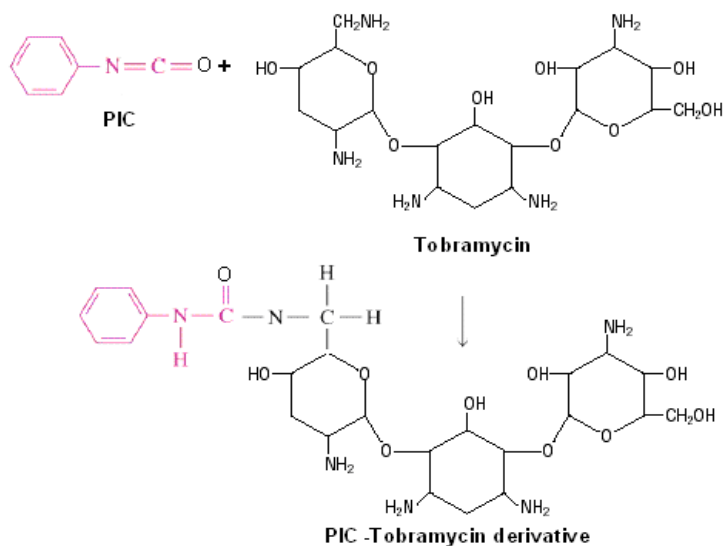
**Figure 1:** Reaction of PIC with tobramycin.

nylon membrane filter 0.45µm pores (Gelman Sciences, Germany) under vacuum. 100µl of the derivatised sample were injected into the HPLC system and separated at ambient temperature using a constant flow rate of 1ml/min.

### 3.1.2. Derivatisation Procedures

In a 4-ml screw-capped glass vial, 1ml of tobramycin aqueous solution was mixed with 1ml of acetnitrile and derivatised with 300µl PIC (1%). Methanol (HPLC-grade) 300µl was added to the reaction mixture to react with the excess amount of PIC reagent, then the mixture was vortex mixed for 40 sec, and heated in water bath at 80° C for 15 minutes. After cooling, 50µl was injected into the HPLC system.

### 3.1.3. Principle of Reaction

Phenylisocyanate (PIC) reacts with both primary and secondary amine in alkaline medium and as well as reacts with hydroxy groups. The reaction is complete within 10 min, and the resulting derivative can be detected by UV absorbance at wavelength 254nm. As shown in Figure **1**, PIC derivatising reagent reacts with the amino group in tobramycin, and other aminoglycosides, to form PIC-tobramycin derivative which is highly detectable compound.

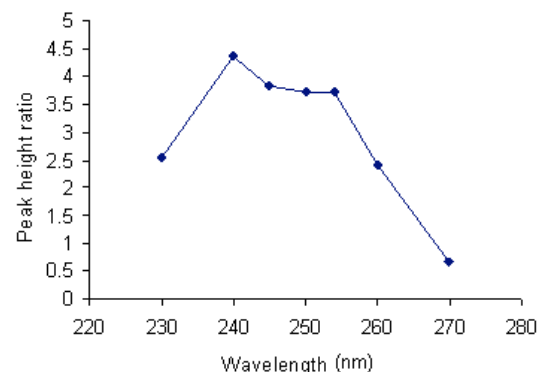### 3.1.4. Optimisation of PIC Derivatisation Method

The derivatisation of tobramycin with PIC derivatising reagent was optimised for reaction time and UV-detection wavelengths. The effect of reaction time was examined by allowing the derivatisation to proceed for various times ranging from 2 to 60 min in a water bath at 80° C. The reaction was completed within

2 min, but the optimal was at 15 min, as shown in Figure **2**.
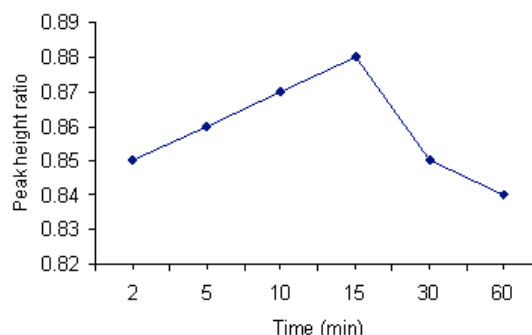


**Figure 2:** Effect of reaction time on PIC-Tobramycin derivative yield.

The UV-detection was examined by detecting the repeated injections of standard tobramycin solution after PIC derivatisation at different wavelengths ranging from 230-270nm. The optimum detection of PIC-tobramycin- derivative was detected at wavelength 240nm, as shown in Figure **3**.



**Figure 3:** The Optimum UV-wavelengths for PIC-tobramycin derivative detection.
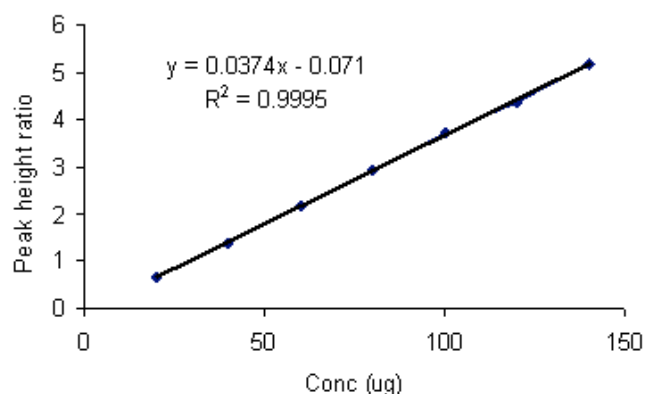
### 3.1.5. PIC Derivatisation Method Validation

#### 3.1.5.1. Linearity

The linearity of the HPLC pre-column PIC derivatisation method was examined by analysis of tobramycin standard solution at seven different concentrations, ranging from 20 to 140µg/ml. As shown in Figure **4**, the standard calibration curve for tobramycin aqueous solution was linear, and described by the following equation:

$$y = 0.0374x - 0.071 \ (n=5)$$

The correlation coefficient in regression line of seven- point calibration was 0.9995.



**Figure 4:** Calibration curve of tobramycin aqueous solution after PIC derivatisation.

#### 3.1.5.2. Sensitivity

The LOD and LOQ of the HPLC pre-column PIC derivatisation method in tobramycin aqueous solution were calculated from the mean of the intercept of five calibration curves. The LOD and LOQ were 0.86 and 2.62µg/ml, respectively.

#### 3.1.5.3. Precision

Reproducibility of the assay in tobramycin aqueous solution was assessed:

- Intra-day by five consecutive analyses of five different concentrations.

- Inter-day also by five consecutive analyses of the same solutions on five days.

The results of Intra-day and Inter-day precision are reported in Table **1**.

The reproducibility of the retention time of PIC-tobramycin derivative was examined during the validation procedure of the analysis method after approximately 50 consecutive injections. The RSD % of the retention time was calculated to be 0.45, 0.389 % (n=50) for tobramycin and neomycin, respectively.

**Table 1: Intra-Day and Inter-Day Precision of Tobramycin Aqueous Solutions after PIC Derivatisation**

| Tobramycin Concentration (µg/ml) | Intra-day | | Inter-day | |
|---|---|---|---|---|
| | STDEV | RSD% | STDEV | RSD% |
| 20 | 0.00198 | 0.411664 | 0.008673 | 1.783705 |
| 40 | 0.00416 | 0.604288 | 0.006004 | 0.871035 |
| 60 | 0.002869 | 0.308969 | 0.007827 | 0.848462 |
| 80 | 0.002401 | 0.213593 | 0.008297 | 0.737566 |
| 100 | 0.002615 | 0.19289 | 0.035418 | 2.56713 |

#### 3.1.5.4. Specificity, Stability and Robustness

The specificity of the assay was examined by testing samples to ensure that there were no interferences from components of the mobile phase, degradation products, other aminoglycosides and amino compounds, and common medications. No interferences were observed with tobramycin peak as shown in Figure **5**.

The stability of PIC-tobramycin derivative was examined by repeated injection of the same sample at different times over a period of 5 days, when stored at room temperature in a clear vial, and by comparing with freshly prepared standard.

The stability of tobramycin solutions was also evaluated at room temperature for 24 hrs. and at 2°C in a refrigerator for 1, 3 and 6 months, then compared to freshly prepared solutions after PIC derivatisation. No significant differences were observed. The robustness of the assay was evaluated by determining the retention times of tobramycin using different lots of the same analytical column, and two different batches of mobile phase solvents.

### 3.1.6. Application of the Developed HPLC Method

#### 3.1.6.1. Determination Amount of Tobramycin in Commercial Dosage Forms

Many commercial dosage forms of tobramycin have been tested for determination amount of tobramycin by the developed HPLC method. The percentage of tobramycin free base in tobramycin sulphate was
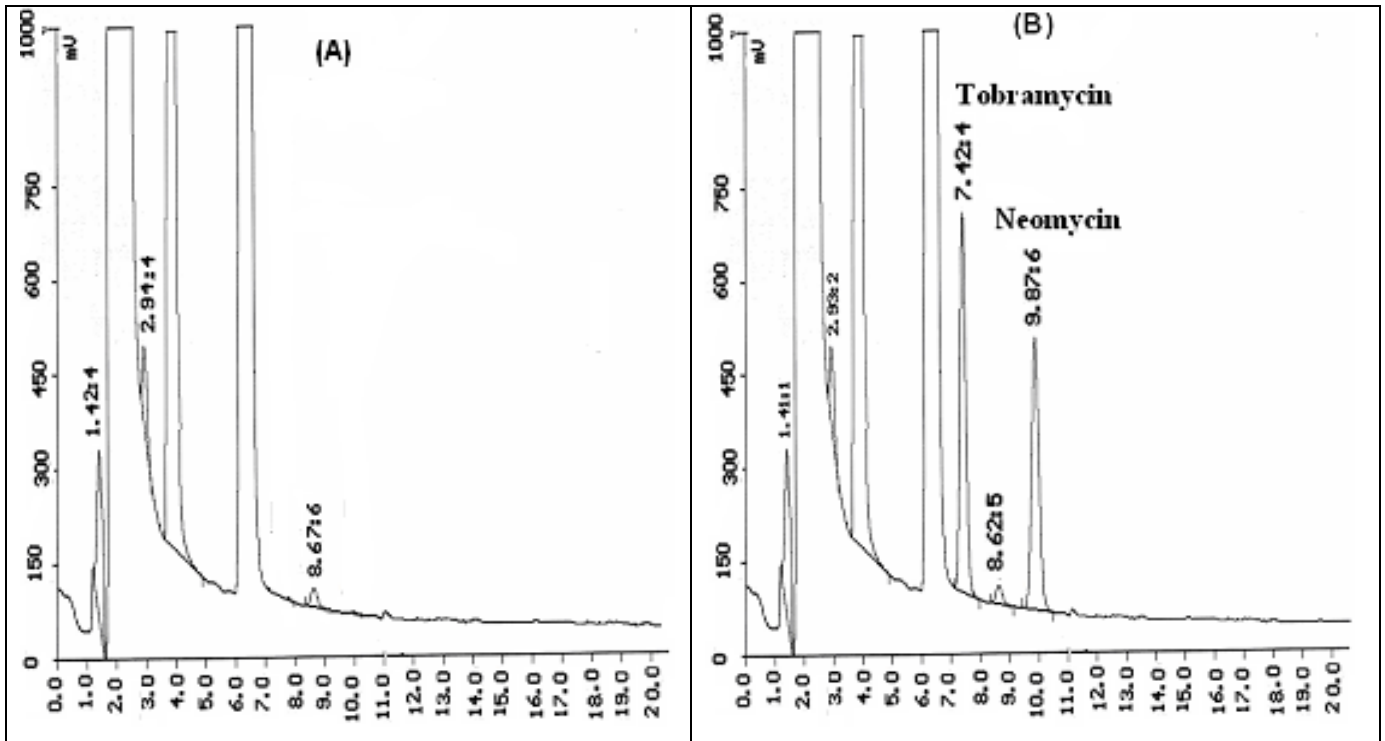
**Figure 5:** Chromatograms obtained for (**A**) a blank (water), (**B**) tobramycin with neomycin (internal standard) after derivatisation with PIC.

detected theoretically and by the proposed HPLC method that is 65.6% so tobramycin sulphate 40mg/ml equivalent to 26,24mg/ml tobramycin free base.

The tested dosage forms include TOBI® (300mg/5 ml, Chiron Corporation, Emeryville, USA), Tobramycin injection B.P 80mg/2ml as Tobramycin sulphate (Cox pharmaceuticals.London.UK), and Tobramycin injection B.P 80mg/2ml as Tobramycin sulphate (David Bull Laboratories, Mulgrave, Victoria, Australia). The analysis assay was further improved by inject standard sample (as a control) inter three consecutive injections for quality control. The analysis data have been shown in Table **2**.

## 3.2. *In vitro* Measurements

### 3.2.1. Study Design

In this study, different nebuliser delivery systems have been used to assess their performance to deliver approved tobramycin inhaled solution 300mg/5ml (TOBI®; Chiron Corporation, Emeryville, USA): two different designs of jet nebulisers and two types of new nebulisers based on vibrating mesh technology. The two jet nebulisers were breath-enhanced Pari LC Plus® jet nebuliser attached to a PariBoy*N*® compressor (Pari GmbH, Starnberg, Germany), and the constant output Sidestream® jet nebuliser (Medic-Aid Ltd, West Sussex, UK) attached to a Porta-Neb® compressor (Profiles, UK). The performance of the Pari LC Plus®

**Table 2: Determination of Amount of Tobramycin in Commercial Dosage Forms, using PIC Derivatisation Method**

| Tobramycin pharmaceutical dosage forms | Actual amount of Tobramycin (mg/ml) | Found amount of Tobramycin (mg/ml) (Mean ±SD) (n=6) |
|---|---|---|
| TOBI® (300mg/5 ml, Chiron Corporation, Emeryville, USA) | 60 | 60 ± 0.057 |
| Tobramycin injection B.P (Cox Pharmaceuticals) | 26.24 (as Tobramycin free base) | 26.20 ± 0.43 |
| Tobramycin injection B.P (David Bull Laboratories) | 26.24 (as Tobramycin free base) | 26 ± 0.12 |

and Sidestream® was also assessed at different gas flow rates: 8L/min, using Medix® AC 2000 Hi Flow compressor (Medix, Ltd., UK), 10 and 12 L/min, using compressed air cylinder. The two types of new nebulisers were Omron® NE-U22 (Omron, Japan) ultrasonic vibrating mesh nebuliser with different frequencies (low, moderate, and high), and Aeroneb® Go (Aerogen, Inc., USA) electronic micropump nebuliser. The nebuliser fill volumes were 5ml and 2.5ml of tobramycin inhaled solution 300mg/5ml (TOBI)® for jet and new nebuliser, respectively. The cascade impactor (Marple 298 series) was used to determine aerosol particle size distribution. The amount of tobramycin extracted from cascade filters was determined by HPLC.

The particle size distribution measurements were calculated as the mean of six determinations. Breathing simulator modelling (Pari Respiratory Equipment, Inc., Germany) was used to determine total inhaled dose, aerosol output rate and percentage of inhaled mass, residual dose volume, and deposited dose. The methodology applied for the determination of the aerosol particle size distribution and output was adapted from the current European standard, EN 13544-1:2001E "Respiratory therapy equipment – Part 1: Nebulising systems and their components (approved by the European Committee for Standardisation CEN in June 2001 and published in August 2001). The European Standard is also a British Standard, BS EN 13544-1:2001 British Standard, October 2001).

The aerosol particle size distribution and aerosol output were determined at ambient conditions (temperature 23±2ºC, relative humidity 45% r.h to 75% r.h, and pressure from 86 kPa to 106 kPa).

### 3.2.2. Data Analysis

The aerosol particle size distribution measurements were calculated as the mean of six determinations. The cumulative mass of nebulized solution retained in the successive stages of the cascade impactor was calculated and plotted on a log-probability scale (as percentage of total mass recovered in the impactor) against the effective cut-off diameter. According to Clark and Borgstrom [19], the experimental mass median aerodynamic diameter (MMAD) of the particles was defined from this graph as the interpolation of the regression line at 50%, by using the Origin 6.1 software program.

The fine particle fraction (FPF), defined as the fraction of the mass-weighted size distribution contained in particles smaller than a specified aerodynamic diameter, typically 5µm, is a descriptive statistic that was directly obtained from the cumulative mass fraction undersize (European Pharmacopoeia, 1997).

The geometric standard deviation (GSD) was calculated as recommended in the draft standard by the equation:

$$GSD = \sqrt{\frac{D_{84.13\%}}{D_{15.87\%}}}$$

The span or volume median diameter (VMD), which is a measure of the width of the volume distribution relative to the median diameter, was calculated by the following equation:

$$Span = \frac{D_{90\%} - D_{10\%}}{D_{50\%}}$$

Span is an index of aerosol polydispersity [20]. The aerosol output measurements, which include inhaled dose in the first minute, aerosol output rate, respirable inhaled mass, respirable inhaled percentage, emitted dose, nebulisation time, and residual volume, were recorded as the mean of ten determinations. The inhalation rate was represented by amount of tobramycin in the first minute. The combination of particle size distribution and output results determined the optimum combinations to deliver approved tobramycin inhaled solution (TOBI)®. The statistical analysis was carried out using the SPSS (version 20) software program. The mean ratio (95% confidence interval) for all tested nebulisers was calculated. The Pari LC Plus® combined with the PariBoy® compressor was reference nebuliser. All tested nebulisers were compared to the reference by using a one-way ANOVA test A mean difference (99.9% confidence interval) was calculated also, and the probability value of p<0.001 was considered to be significant.

## 4. RESULTS AND DISCUSSIONS

Nebuliser aerosol delivery is largely dependent on the nebuliser design and the physicochemical properties of drug solution. In this chapter, the effect of nebuliser design on improving tobramycin aerosol delivery has been studied by using different nebuliser delivery systems. The effect of changes in physicochemical properties of drug solution will be discussed in the next chapter. Different nebuliser delivery systems have been assessed to determine the optimum combination to deliver TOBI® solution and to

overcome the medication high cost problem by determining the best nebuliser system, characterised by high aerosol delivery and less drug wastage.

Two different designs of jet nebulisers and new nebulisers based on vibrating mesh technology have been used. The constant output Sidestream[®] and breath-enhanced Pari LC Plus[®] jet nebulisers were used in combination with different compressors at different flow rates. The gas flow rate is the major determinant of the aerosol droplet size and the aerosol output of jet nebulisers. Thus, the influence of gas flow rate on tobramycin aerosol droplets size and output has been examined. The Sidestream[®] was combined with PotaNeb[®] compressor (flow rate 6.8 L/min), and Pari LC Plus[®] combined with PariBoy*N*[®] compressor (flow rate 7 L/min). Both the Sidestream[®] and Pari LC Plus[®] nebulisers were also combined with Medix[®] AC 2000 Hi Flow compressor (flow rate 8 L/min) and compressed air cylinder, at flow rate 10 and 12 L/min. The analysis of particle size distribution measurements showed that the MMAD values decreased as air jet flow rate increased from 6.8-12 L/min, while the %FPF and VMD values were proportional to air jet flow rate. The mean (SD) of the smallest MMAD values in tested jet nebulisers was 1.22 (0.04), 1.33 (0.02) µm for Sidestream[®] and Pari LC Plus[®], respectively, in combination with compressed air at flow rate 12L/min.

The mean (SD) highest %FPF in tested jet nebulisers was 78 (1.11), 70 (1.13) % for Sidestream[®] and Pari LC Plus[®], respectively, also in combination with compressed air at flow rate 12L/min. The difference in the results between both jet nebuliser systems was due to the difference in the internal design of the nebuliser. When comparing different frequencies (low, moderate and high) of NE-U22 Omron[®] ultrasonic vibrating mesh nebuliser, the smallest droplet size was produced by moderate frequency. However, all tested nebulisers were produced droplets within the respirable size (2-5µm), as shown in Figure **6**, but the smallest particle size was observed for NE-U22-E Omron[®] moderate frequency by mean (SD) 1.18 (0.03).

The percentage of droplets less than 5µm was used to define the respirable (peripheral) percentage. The mean (SD) of highest droplets percentage (<5µm) was 82.21 (1.72) % also for NE-U22-E Omron[®] moderate frequency, while the least droplets percentage value was 57.3 (2.58) % for Pari LC Plus[®] combined with PariBoy*N*[®] compressor. In general, the heterodispersity of aerosol produced from jet nebulisers was greater than the vibrating mesh nebulisers.

All nebulised aerosol droplets were polydispersed with span values ranging between 2.93-5.37µm for jet nebulisers and 1.76-2.21µm for vibrating mesh nebulisers. The particle size distribution measurements are summarised in Table **3**. The aerosol output was also affected by gas flow rate in jet nebulisers, the high flow rate leading to rapid evaporation of solvent and rapid decrease in the temperature of the fluid within the nebuliser, approximately 10 -15 ° C [21, 23]. This may result in changes in the physiochemical properties of nebuliser fluids and precipitation of drug in the nebuliser [23]. The analysis data of aerosol output showed that the Medix[®] AC 2000 Hi Flow compressor (flow rate 8 L/min) was the optimum combination with both Sidestream[®] and Pari LC Plus[®] jet nebulisers which produced highest aerosol output when compared at different gas flow rates.
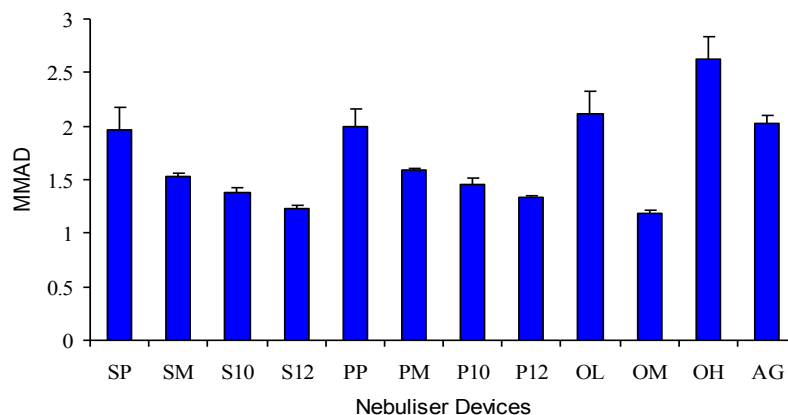


**Figure 6:** Mean (SD) MMAD obtained by different nebuliser systems during nebulisation of TOBI[®] solution.

Key**:** P12 =Pari LC Plus[®] /air cylinder at flow rate 10 and 12 OL, OM and OH = low, moderate and high frequency of NE-U22 Omron[®], and AG= Aeroneb[®] Go.

**Table 3:  Summary of Means (n=6) Aerosol Particle Size Distribution Measurements during Nebulisation of TOBI®
Solution by Selected Nebuliser Delivery Systems**

| Nebuliser Device | MMAD µm | | GSD µm | | Fine Particles <5 µm (mg) | | % FPF | | VMDµm | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean (SD) | 95% CI | Mean (SD) | 95% CI | Mean (SD) | 95% CI | Mean (SD) | 95% CI | Mean (SD) | 95% CI |
| Sidestream®/ Porta-Neb | 1.96± 0.2 | 1.72 – 2.19 | 2.2 ± 0.05 | 2.17 – 2.27 | 1.5 ± 0.16 | 1.30 – 1.64 | 60.6±4.05 | 56.4 – 64.9 | 2.9±0.3 | 2.61 – 3.24 |
| Sidestream®/ Medix AC 2000 | 1.5±0.03 | 1.47 – 1.57 | 2.7 ±0.07 | 2.59 – 2.82 | 3.29±0.16 | 3.0 – 3.55 | 64.6±1.04 | 62.9 – 66.2 | 4.1±0.12 | 3.94 – 4.31 |
| Sidestream®/ Air 10L/min | 1.38±0.05 | 1.29 – 1.45 | 2.88±0.04 | 2.82 – 2.94 | 4.18±0.26 | 3.76 – 4.59 | 69±2.12 | 65.6 – 72.2 | 4.16±0.23 | 3.79 – 4.52 |
| Sidestream®/ Air 12L/min | 1.22±0.04 | 1.17 – 1.26 | 2.42±0.13 | 2.28 – 2.55 | 5.03±0.17 | 4.85 – 5.20 | 78.13± 1.1 | 76.9 – 79.3 | 3.41±0.17 | 2.70 – 3.79 |
| Pari LC Plus®/ PariBoy | 1.99±0.17 | 1.82 – 2.17 | 2.67±0.14 | 2.53 – 2.81 | 2.2±0.23 | 1.95 – 2.43 | 57.3±2.58 | 54.6 – 60.0 | 3.48±0.23 | 3.23 – 3.71 |
| Pari LC Plus®/ Medix AC 2000 | 1.59±0.02 | 1.56 – 1.61 | 2.72±0.15 | 2.56 – 2.87 | 6.5±0.55 | 5.92 – 7.07 | 64.4±1.32 | 63.0 – 65.8 | 5.33±0.3 | 4.99 – 5.67 |
| Pari LC Plus®/Air 10L/min | 1.45±0.06 | 1.42 – 1.57 | 2.82±0.25 | 2.50 – 3.12 | 5.5±0.72 | 4.60 – 6.39 | 65.4±2.9 | 61.8 – 69.0 | 4.35±0.34 | 3.92 – 4.76 |
| Pari LC Plus®/ Air 12L/min | 1.33±0.02 | 1.31 – 1.35 | 2.96 ±0.06 | 2.90 – 3.02 | 8.68±0.81 | 7.82 – 9.53 | 70.04±1.13 | 68.8 – 71.2 | 5.37±0.20 | 5.19– 5.53 |
| NE-U22 Omron® / LF | 2.12±0.2 | 1.89 – 2.34 | 1.76 ±0.06 | 1.69 – 1.83 | 1.20 ± 0.1 | 1.09 – 1.30 | 60.36±5.38 | 54.7 – 66.0 | 1.74±0.17 | 1.56 – 1.91 |
| NE-U22-E Omron® / MF | 1.18±0.03 | 1.14 – 1.21 | 1.99 ±0.08 | 1.90 – 2.08 | 3.44 ±0.26 | 3.16 – 3.71 | 82.21±1.72 | 80.4 – 84.0 | 2.60±0.18 | 2.39 – 2.78 |
| NE-U22 Omron® / HF | 2.63± 0.2 | 2.42 – 2.84 | 2.21±0.04 | 2.16 – 2.24 | 3.47 ±0.33 | 3.13 – 3.81 | 48.81±2.70 | 45.9 – 51.6 | 2.44±0.1 | 2.34 – 2.53 |
| Aeroneb Go® | 2.02±0.08 | 1.93 – 2.10 | 2.11±0.07 | 2.03 – 2.18 | 2.81±0.25 | 2.54 – 3.07 | 59.9 ± 1.4 | 58.4 – 61.4 | 2.62±0.08 | 2.53 – 2.70 |

95% CI=Confidence Intervals. MMAD=Mass Median Aerodynamic Diameter, GSD= Geometric Standard Deviation, % FPF =Fine particle Fraction Percentage,
VMD=Volume Median Diameter

All new nebulisers, NE-U22 Omron® (low, moderate, and high frequency) and Aeroneb Go®, showed a high percentage of respirable inhaled mass with fill volume less than jet nebulisers. The mean (SD) of highest percentage of respirable inhaled mass was 43 (1.15) % for the NE-U22-E Omron® (moderate frequency) nebuliser. The drug wastage was increased with jet nebulisers due to evaporation of solvent during jet nebulisation leading to a gradual increase in the concentration of drug solution left behind. For economic reasons, the mean (SD) of highest drug wastage percentage was 61.95 (1.78) % for the Sidestream® jet nebuliser combined with PotaNeb® compressor, while the mean (SD) least drug wastage percentage was 1.28 (0.27) % for the NE-U22-E Omron® (moderate frequency) nebuliser. In general, the drug wastage in vibrating mesh nebulisers was less than in jet nebulisers. The aerosol output characteristics are summarised in Table **4**. All tested nebulisers were compared to Pari LC Plus®, which is recommended by the manufacturer of TOBI®,

combined with PariBoy*N*® compressor, by using a one-way ANOVA test.

The statistic data showed that the MMAD values were significantly decreased (p< 0.001) by increasing the driven gas flow rate in both Pari LC Plus® and Sidestream® jet nebulisers. The fine particle dose and span values were significantly increased (p< 0.001) by increasing the gas flow rate in both jet nebulisers. When comparing the vibrating mesh nebulisers to the Pari LC Plus®/ PariBoy*N*® combination, the droplet size was significantly decreased (p< 0.001), while %FPF was significantly increased (p< 0.001) in NE-U22 Omron® (moderate and high frequency) nebulisers. In vibrating mesh nebulisers, the percentage of respirable inhaled mass was significantly increased (p< 0.001), while the percentage of residual volume was significantly decreased (p< 0.001). Comparisons of tested nebulisers to Pari LC Plus® combined with PariBoy*N*® compressor are summarised in Tables **5** and **6**. When the results data of the particle size distribution and output measurements are combined, it

**Table 4:**   **Summary of Means (n=10) Aerosol Output Measurements during Nebulisation of TOBI® Solution by Selected Nebuliser Delivery Systems**

| Nebuliser Device | Fill Volume (ml) | Nebulisation Time (min) | | Inhalation Rate (mg/min) | | Respirable inhaled Mass (mg) | | % Respirable inhaled Mass | | % Residual of Dose Volume | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean (SD) | 95% CI | Mean (SD) | 95% CI | Mean (SD) | 95% CI | Mean (SD) | 95% CI | Mean (SD) | 95% CI |
| Sidestream®/ Porta-Neb | 5 | 15±0.2 | 14.8-15.1 | 3.5 ± 0.2 | 3.4 - 3.7 | 32.3 ±2.1 | 30.8-33.7 | 10.7 ±0.7 | 10.2 - 11.2 | 61.9 ± 1.8 | 60.6-63.2 |
| Sidestream®/ Medix AC 2000 | 5 | 13.3±0.5 | 12.6-13.9 | 6.6 ± 0.1 | 6.4 - 6.8 | 56.8 ±1.2 | 55.3-58.3 | 18.9 ±0.4 | 18.4-19.4 | 36.2 ±0.7 | 35.3-37.2 |
| Sidestream®/ Air 10L/min | 5 | 10±0.14 | 9.8 - 10.2 | 8.2 ± 0.2 | 7.9 - 8.5 | 56.9 ±1.5 | 55- 58.8 | 18.9 ±0.5 | 18.3-19.6 | 37.2 ±0.9 | 36.1-38.4 |
| Sidestream®/ Air 12L/min | 5 | 8.5±0.16 | 8.3 - 8.6 | 5.6 ± 0.3 | 5.4 - 5.8 | 37.3±1.82 | 36 - 38.6 | 12.4 ± 0.6 | 12 - 12.8 | 56.3 ±2.1 | 54.8-57.9 |
| Pari LC Plus®/ PariBoy | 5 | 13±0.33 | 12.9 - 13.2 | 6.6 ± 0.3 | 6.4 - 6.9 | 49.5 ± 2.5 | 47.6-51.3 | 16.5 ±0.8 | 15.9-17.1 | 41.2 ±2.1 | 39.7-42.7 |
| Pari LC Plus®/ Medix AC 2000 | 5 | 11.3±0.3 | 10.9 - 11.6 | 7.2±0.6 | 6.5 - 7.9 | 52.5 ± 4.2 | 47.2-57.8 | 17.5 ±1.4 | 15.7-19.2 | 38.3 ± 7.8 | 28.5-48.1 |
| Pari LC Plus®/ Air 10L/min | 5 | 10.7±0.1 | 10.5 - 10.8 | 7.7±0.42 | 7.2 - 8.2 | 54.2 ± 2.9 | 50.6-57.8 | 18.1±0.97 | 16.8-19.3 | 42.8 ±3.9 | 37.9-47.7 |
| Pari LC Plus® /Air 12L/min | 5 | 9±0.21 | 8.7 - 9.26 | 8.17±0.2 | 7.8 - 8.4 | 51.5 ±1.5 | 49.6-53.3 | 17.2 ±0.5 | 16.5-17.7 | 39.8 ±1.5 | 37.9-41.6 |
| NE-U22 Omron® / LF | 2.5 | 20±0.36 | 19.7 - 20.2 | 3.3±0.1 | 3.2 - 3.4 | 39.8 ± 1.6 | 38.6-40.9 | 26.5 ±1 | 25.7-27.3 | 4.7 ±0.38 | 4.42 – 4.9 |
| NE-U22-E Omron® / MF | 2.5 | 15.4±0.2 | 15.3 - 15.6 | 5.11±0.1 | 5.0 - 5.2 | 64.9 ±1.7 | 63.6-66.1 | 43.2 ±1.1 | 42.4-44.1 | 1.3 ±0.3 | 1.1 - 1.47 |
| NE-U22 Omron® / HF | 2.5 | 8.2±0.17 | 8.1 - 8.3 | 7.81±0.2 | 7.6 - 7.9 | 38.8 ±1 | 38.1-39.5 | 25.9±0.67 | 25.4-26.4 | 5.1 ± 0.3 | 4.9 - 5.2 |
| Aeroneb Go® | 2.5 | 7 ± 0.09 | 6.97. 06 | 10.04±1.7 | 9.7 - 10.3 | 42.1 ±1.9 | 40.7-48.6 | 28.1 ± 1.3 | 27.1-28.9 | 25.9 ± 1.9 | 24.5-27.3 |

95% CI= Confidence Intervals.
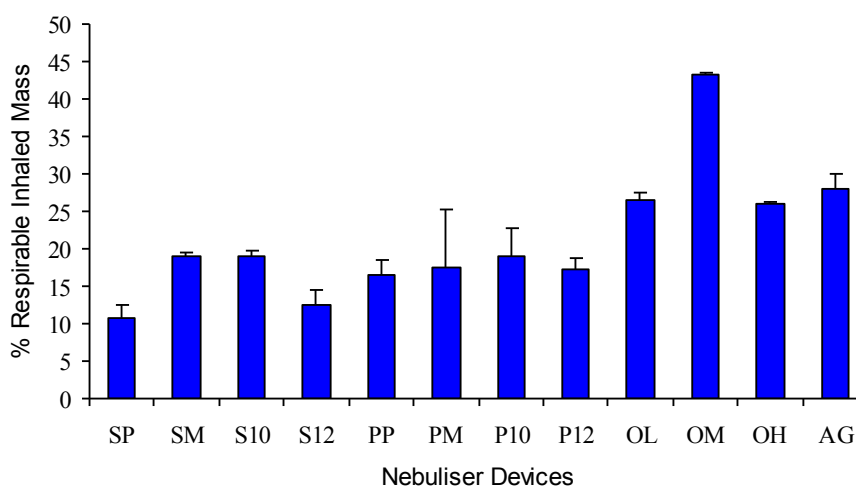


**Figure 7:** Mean (SD) respirable inhaled mass percentage of tobramycin produced by selected nebuliser systems during nebulisation of a TOBI® solution.

can concluded that the moderate frequency NE-U22-E Omron® and Aeroneb Go® are preferred to nebulise TOBI® solution. The NE-U22-E Omron® moderate frequency showed the smallest aerosol droplets size, highest respirable droplets percentage, highest respirable inhaled mass, the least drug wastage, and reasonable nebulisation time, while Aeroneb Go® showed the shortest nebulisation time.
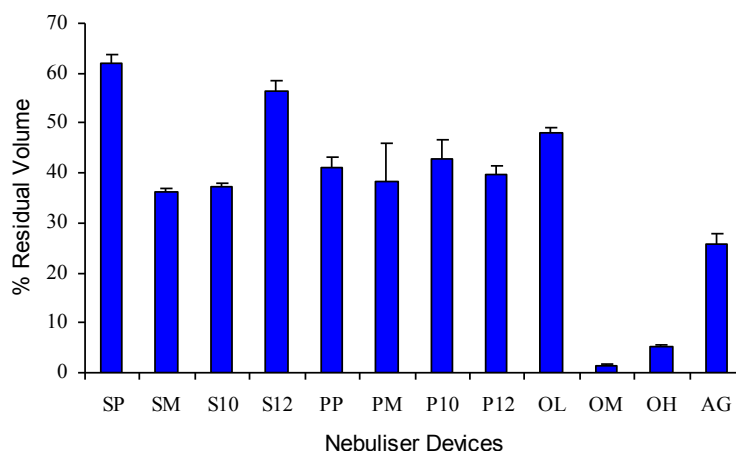
**Figure 8:** Mean (SD) residual volume percentage of tobramycin after nebulisation of a TOBI® solution by selected nebuliser systems.

**Table 5:** **Mean Difference (99.9% CI) of Particle Size Measurements Obtained by Selected High Performance Nebuliser Systems Compared to Recommended Pari LC Plus® Jet Nebuliser Combined with PariBoy*N*®, using One-Way ANOVA Test (Tukey HSD)**

| | MMAD µm | | | GSD µm | | | Fine Particles <5 µm (mg) | | | % FPF | | | VMD µm | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MD | 99.9% CI | | MD | 99.9% CI | | MD | 99.9% CI | | MD | 99.9% CI | | M D | 99.9% CI | |
| | | LB | UB | | LB | UB | | LB | UB | | LB | UB | | LB | UB |
| Sidestream®/ Porta-Ne | 0.038 | -0.31 | 0.38 | 0.450* | 0.148 | 0.750 | 0.724 | -0.37 | 1.82 | -3.350 | -10.61 | 3.903 | 0.557 | -0.15 | 1.27 |
| Sidestream®/ Medix AC | 0.474* | 0.086 | 0.86 | -0.037* | -0.373 | 0.298 | -1.089 | -2.313 | 0.134 | -7.287 | -15.39 | 0.821 | -0.643 | -1.44 | 0.15 |
| Sidestream®/ Air 10L/min | 0.624* | 0.24 | 1.012 | -0.209 | -0.545 | 0.127 | -1.979* | -3.203 | -0.755 | -11.685* | -19.79 | -3.576 | -0.677* | -1.47 | 0.12 |
| Sidestream®/ Air 12L/min | 0.775* | 0.429 | 1.122 | -0.257 | -0.043 | 0.558 | -2.831* | -3.925 | -1.74 | -20.810* | -28.05 | -13.55 | 0.070 | -0.52 | 0.66 |
| Pari LCPlus®/ Medix AC | 0.412* | 0.065 | 0.76 | -0.045 | -0.346 | 0.255 | -4.297* | -5.392 | -3.203 | -7.093 | -14.34 | 0.159 | -1.846* | -2.56 | -1.13 |
| Pari LC Plus®/ Air 10L/min | 0.499* | 0.136 | 0.86 | -0.139 | -0.455 | 0.175 | -3.301* | -4.44 | -2.153 | -8.103* | -15.71 | -0.496 | -0.862* | -1.61 | -0.12 |
| Pari LC Plus®/ Air 12L/min | 0.665* | 0.318 | 1.012 | -0.285 | -0.585 | 0.015 | -6.480* | -7.574 | -5.385 | -12.720* | -19.97 | -5.47 | -1.879* | -2.59 | -1.17 |
| NE-U22 Omron®/ LF | -0.121 | -0.47 | 0.23 | 0.915* | 0.614 | 1.215 | 0.996 | -0.099 | 2.089 | -3.036 | -10.23 | 4.216 | 1.746* | 1.03 | 2.46 |
| NE-U22-E Omron® / MF | 0.820* | 0.472 | 1.16 | 0.681* | 0.380 | 0.982 | -1.244* | -2.339 | -0.149 | -24.890* | -32.14 | -17.63 | 0.899* | 0.19 | 1.61 |
| NE-U22 Omron®/ HF | -0.637* | -0.98 | -0.290 | 0.467* | 0.166 | 0.767 | -1.275* | -2.370 | -0.181 | 8.511* | 1.258 | 15.76 | 1.045* | 0.33 | 1.76 |
| Aeroneb Go® | -0.021 | -0.37 | 0.325 | 0.568* | 0.267 | 0.870 | -0.613 | -1.707 | 0.482 | -2.610 | -9.861 | 4.643 | 0.865* | 0.15 | 1.58 |

99.9% CI=Confidence Intervals.
LB=Lower Bound, UB=Upper Bound.
MD=Mean Difference.
*Mean difference significant at 0.001 level (p<0.001).

## 5. CONCLUSIONS

A simple, accurate and sensitive HPLC method for the determination of tobramycin followed by phenylisocyanate (PIC) pre-column derivatisation has been developed.

The good validation results of the proposed method show the suitability to use it in the clinical and research laboratories for the measurement of tobramycin and other aminoglycosides in biological fluids or pharmaceutical formulations. The main advantages of the proposed method are the high sensitivity and

**Table 6:** **Mean Difference (99.9% CI) of Output Measurements Obtained by Selected high Performance Nebuliser Systems Compared to Recommended Pari LC Plus® Jet Nebuliser Combined to PariBoy*N*® Compressor, using One-Way ANOVA Test (Tukey HSD)**

| | Nebulisation Time (min) | | | Inhalation Rate (mg/min) | | | Respirable inhaled Mass (mg) | | | % Respirable inhaled Mass | | | % Residual of Dose Volume | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MD | 99.9% CI | | MD | 99.9% CI | | MD | 99.9% CI | | MD | 99.9% CI | | MD | 99.9% CI | |
| | | LB | UB | | LB | UB | | LB | UB | | LB | UB | | LB | UB |
| Sidestream®/ Porta-Ne | -1.930* | -2.43 | -1.43 | 3.096* | 1.805 | 4.388 | 17.22* | 13.034 | 21.420 | 5.742* | 3.872 | 7.613 | -20.739* | -25.64 | -15.83 |
| Sidestream®/ Medix AC | -0.230 | -0.84 | 0.381 | 0.035 | -1.546 | 1.617 | -7.278* | -12.414 | -2.143 | -2.426* | -4.717 | -0.135 | 4.959 | -1.047 | 10.966 |
| Sidestream®/ Air 10L/min | 3.070* | 2.46 | 3.681 | -1.603* | -3.185 | -0.021 | -7.402* | -12.537 | -2.267 | -2.467* | -4.758 | -0.176 | 3.954 | -2.052 | 9.962 |
| Sidestream®/Air 12L/min | 4.570* | 4.07 | 5.068 | 1.025 | -0.266 | 2.317 | 12.20* | 8.010 | 16.393 | 4.067* | 2.196 | 5.937 | -15.143* | -20.05 | -10.23 |
| Pari LCPlus®/ Medix AC | 1.770* | 1.16 | 2.381 | -0.572 | -2.154 | 1.010 | -3.013 | -8.150 | 2.121 | -1.004 | -3.874 | 1.286 | 2.913 | -3.093 | 8.920 |
| Pari LC Plus®/ Air 10L/min | 2.370* | 1.76 | 2.981 | -1.106 | -2.688 | 0.475 | -4.750 | -9.885 | 0.385 | -1.583 | -3.874 | 0.707 | -1.664 | -7.672 | 4.34 |
| Pari LC Plus®/ Air 12L/min | 4.070* | 3.46 | 4.681 | -1.523 | -3.105 | 0.059 | -1.972 | -7.107 | 3.162 | -0.657 | -2.948 | 1.633 | 1.396 | -4.611 | 7.430 |
| NE-U22 Omron® / LF | -6.930* | -7.43 | -6.431 | 3.348* | 2.057 | 4.640 | 9.730* | 5.536 | 13.922 | -10.018* | -11.88 | -8.147 | 36.51* | 31.65 | 41.37 |
| NE-U22-E Omron® / MF | -2.380* | -2.88 | -1.881 | 1.546* | 0.245 | 2.828 | -15.36* | -19.555 | -11.170 | -26.745* | -28.62 | -24.87 | 39.924* | 35.02 | 44.83 |
| NE-U22 Omron® / HF | 4.870* | 4.37 | 5.368 | -1.165 | -2.456 | 0.127 | 10.66* | 6.467 | 14.853 | -9.397* | -11.26 | -7.526 | 36.135* | 31.23 | 41.040 |
| Aeroneb Go® | 6.070 | 5.57 | 6.568 | -3.39* | -4.00 | -2.32 | 7.400* | 3.207 | 11.593 | -11.570* | -13.44 | -9.699 | 15.283* | 10.38 | 20.187 |

99.9% CI=Confidence Intervals.
LB=Lower Bound, UB=Upper Bound.
MD=Mean Difference.
*Mean difference significant at 0.001 level (p<0.001).

stability of PIC-tobramycin derivative and short run time of analysis.

The performance of recent selected nebulisers evaluated to nebulise tobramycin inhaled solution, using the HPLC developed method. The NE-U22 Omron® moderate frequency and Aeroneb Go® and were the best that are greater inhaled mass, less residual volume and short treatment time.

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