Rubidium Efflux Assay for the Determination of Calcium Activated Potassium Channel Activity

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Abstract
Rubidium efflux assay using flame atomic absorption spectrometry is employed in analyzing potassium channel activity. Calibration using standards of known $\text{Rb}^+$ concentrations (10 - 100µM) in tubes was done at the beginning and end of each analysis. $\text{Rb}^+$ standard curves were constructed from the data obtained from the analysis of the $\text{Rb}^+$ standards in both the tubes and 96 well plates. HEK293 cells expressing the alpha subunit of BK channel were incubated with $\text{Rb}^+$ for 4 hours after which the cells were washed and then treated with higher concentrations of KBS (50mM / 80mM) or NS1619 (0.003 - 100µM) for 10 minutes. The supernatant was removed and the cells lysed with 0.1%v/v triton. The percentage efflux was then determined from values obtained after analyzing the supernatant and lysate using flame atomic absorption spectrometer. The results showed that there was consistency during each analysis as the $\text{Rb}^+$ standard curves constructed from the data obtained overlapped with no significant difference indicating precise calibration and internal validation. For the loaded cells (untreated), the average concentration of $\text{Rb}^+$ in the supernatant was 14.47µM while that in the lysate was 56.24µM and statistical analysis showed there was a significant difference with $p<0.0001$. The treated cells with higher concentrations of KBS in comparison with 5.4mM KBS gave a percentage increase in $\text{Rb}^+$ efflux of 47.8% for 50mM KBS with significant different of $p<0.0001$ and 80.11% increase with significant different of $p<0.05$ for 80mM KBS. The treated cells with 0.1, 0.01 and 0.003µM NS1619 gave a percentage increase in efflux of 13.98%, 29.95% and 23.69% respectively. This research indicated the viability of using flame atomic absorption spectrometry for rubidium efflux assay to test for compounds activating effect on BK channel.

Abbreviation: BK (calcium activated potassium channels), Rubidium ion ($\text{Rb}^+$), Human embryonic kidney cells (HEK293), potassium chloride buffer saline (KBS), NS1619(1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3H) benzimidazolone)

Keywords: Rubidium efflux, Flame atomic absorption spectrometry, BKα channel, HEK293 cells, NS1619, 50mM and 80mM KBS.

1. Introduction
Calcium activated potassium (BK) channels are potassium channels that are both voltage and calcium regulated (Ge et al., 2014). They are found in several mammalian cells such as the smooth muscle cells, brain, cochlea, pancreatic islets, endothelial cells and bladder (Ghatta et al., 2006; Ge et al., 2014). They are involved in the regulation of some physiological functions such as vascular tone, neuronal vascular coupling, neuronal excitability, neurotransmitter release, retinal circulation and urinary potassium ion excretion making them important in understanding the physiology of some conditions such as Alzheimer’s disease, neuromuscular...
abnormality, motor impairment, epilepsy, overactive urinary bladder, hypertension, subarachnoid hemorrhage, noise induced hearing loss, diabetes and urinary incontinence (Vetri et al. 2014).

BK channel openers are compounds that increase the permeability of the plasma membrane resulting to an increase in potassium ions efflux with subsequent decrease in cell excitability and smooth muscle relaxation (Ghatta et al. 2006). They have a potential therapeutic effect in conditions such as angina pectoris, hypertension, congestive cardiac failure and asthma (Szewczyk et al 2010; Wu et al. 2014). NS1619, NS1604, NS004, NS11021 and NS1643 are some examples of synthetic BK channel openers. Furthermore, NS1619 was first found to cause hyperpolarization in aortic smooth muscles by activating the BK channel (Olsen et al. 1994). However, NS11021 directly interacts with mitochondria leading to specific potassium ion flux (Szewczyk et al 2010; Wu et al. 2014). The BK channel activation potency of these synthetic channel openers is not absolutely related to their benzimidazolone structure as some other indolone compounds such as BMS 204352 and CGS 7181 have BK activation potency (Ghatta et al. 2006). Mefenamic and flufenamic acids are also examples of synthetic BK channel openers (Ghatta et al. 2006). According to Bentzen et al. (2014), there is no particular synthetic BK channel opener that is in use therapeutically on patients and only Andolast is in clinical development. This research will therefore aid in providing more BK channel modulatorsto be available for clinical trial and for future use in the treatment of some conditions.

Rubidium efflux assay using flame atomic absorption spectrometry is a medium throughput assay that is used in determining the activity of the potassium channel. It involves the use of a non-radioactive rubidium as a measure for potassium ion flux. The method is easy to perform and inexpensive (Mckay et al., 2008) and requires standards with known concentrations to ascertain the relationship between the measured absorbance and the concentration of the standard in a sample.

The objectives of this research are to confirm the reliability and accuracy of flame atomic absorption spectrometer by constructing a standard curve of the absorbances against the concentrations obtained from the analysis of known Rb⁺ concentrations and also to verify the stimulating effect of higher concentrations of potassium chloride (50mM and 80mM) and various concentrations of NS1619. However, the overall aim is to validate rubidium efflux assay using flame atomic absorption spectrometry for determining the modulating effects of compounds on potassium channel activity.

In this research, HEK293 cells expressing the α subunit of BK channel was cultured, loaded with rubidium, treated with some compounds and the Rubidium efflux determined by measuring the Rubidium content of both the supernatant and the lysate using flame atomic absorption spectrometer.

2. Materials and Methods

Cell Culture

Experiments were done on Human embryonic kidney (HEK293) expressing the α-subunit of the human BKca channel which were cultured in 75cm² flasks containing Dulbecco’s modified eagles media (DMEM) supplemented with 10% fetal calf serum and 0.5 µg/µl G418 sulphate and incubated at 37°C in 5% CO₂ humidified incubator.

Upon reaching 95% confluent cell growth, the spent media was poured off and 10mL of PBS was added to rinse the cells. The PBS solution was then poured off and 2mL of trypsin/EDTA solution was added. The flask was then incubated for 2 minutes; after which it was tapped to dislodge the cells. DMEM (5mL) was then added and the content transferred into a falcon tube. It was then centrifuged at 1000rpm for 5 minutes. The supernatant was then discarded and the pellet resuspended in 10mL of DMEM. Cells were seeded for culturing in 75cm² flasks at a 1:10 dilution by adding 1mL of the cell suspension and 25mL of the prepared media.

The remaining cells in the cell suspension were counted using a haemocytometer and 20mL of a cell suspension containing 100,000 cells/mL was prepared. The cells were then seeded (20,000 cells/well) onto a 96-well plate in DMEM containing 10% FCS and G418 sulphate and grown until confluent for 48 hours. Prior to experiments, the cell culture media was removed from each well and the cells were washed with PBS (200µl/well).

Preparation of Buffers, Solutions and NS1619

All solutions were prepared using distilled, deionized water and in clean good quality glassware.

- A stock solution of potassium buffer solution (KBS) was prepared containing 5.4mM KCl, 150mM NaCl, 1mM MgCl₂, 0.8mM NaH₂PO₄, 2mM CaCl₂, 25mM HEPES and 5mM glucose. The pH was adjusted with conc HCl to 7.2 and the solution stored at 4°C.

- Solution of 50mM KBS was prepared containing 50mM KCl, 150mM NaCl, 1mM MgCl₂, 0.8mM NaH₂PO₄, 2mM CaCl₂, 25mM HEPES and 5mM glucose. The pH was adjusted with conc HCl to 7.2 and the solution stored at 4°C.
Solution of 80mM KBS was prepared containing 80mM KCl, 150mM NaCl, 1mM MgCl₂, 0.8mM NaH₂P₂O₄, 2mM CaCl₂, 25mM HEPES and 5mM glucose. The pH was adjusted with conc HCl to 7.2 and the solution stored at 4°C.

Stock solution of Rubidium buffer solution (RBS) was prepared containing 5.4mM RbCl, 150mM NaCl, 1mM MgCl₂, 0.8mM NaH₂P₂O₄, 2mM CaCl₂, 25mM HEPES and 5mM glucose. The pH was adjusted with conc HCl to 7.2 and the solution stored at 4°C.

Triton X-100 lysis solution containing 0.1%v/v of triton X-100 in KBS was prepared and the solution stored at 4°C.

Stock solution of 1mM NS1619 in DMSO was prepared and diluted with KBS to prepare 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0.003 µM solutions.

Flame Atomic Absorption Spectrometry: Set-up and Calibration
The nebulizer port and burner slit of the flame atomic absorption spectrometer (FAAS) were cleaned with a metal wire. The gas and air supply to the instrument were turned on. The instrument and the associated PC running the software were then turned on. The flame was ignited and the instrument was allowed to warm up for 20 minutes. The method on the software was set for the concentrations of the standards to be used.

The standard solutions of RbCl were put into falcon tubes and placed in the sampler rack in the following orientation: optimizing standard (100 µM), blank (de-ionized water), 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µM RbCl respectively. Each standard (300µL) solution was then put into the wells of the first row of a 96-well plate. A calibration curve was constructed from the readings of the standard solutions in the tubes (at the beginning and end of the analysis) and the absorbances of the standards in the 96-well plates determined using the FAAS instrument.

Rubidium (Rb⁺) Efflux Assay
2.2.4.1. Loaded cells and Washes (un-stimulated)
The spent media from each well of 96-well plate that has been seeded and cultured as above (cell culture) was removed by means of a multichannel pipette. The cells monolayer was washed by addition and removal of 200µL PBS. To each well, 200µL of RBS was added and incubated for 4 hours. The cells were then washed four times with KBS (200µl/well). The last wash (supernatant) was transferred to a new 96-well plate and the cells in the first 96-well plate were lysed with 0.1%v/v Triton X-100 in KBS (200 µl/ well) for 18 hours. The volume of each well was made up to 300µl by adding 100µl KBS. Rubidium content was determined in both the supernatant and lysate samples with the standards of RbCl using the flame atomic absorption spectrometer.

Cells treated with higher concentrations of KCl (KBS)
The cells were incubated in RBS (200 µl/ well) for 4 hours, then washed four times with KBS (200 µl/well) as above. Half of the 96-well plate containing the cells was then exposed to 5.4mM KBS (200 µl/well) while the remaining half with 50mM KBS (200 µl/well) for 10-min. The supernatant was transferred to a new 96-well plate and the cells lysed with 0.1% Triton X-100 in KBS (200 µl/ well) for 18 hours. The volume of each well was made up to 300µl by adding 100µl KBS. Rubidium content was determined in both the supernatant and lysate samples with the standards of RbCl using the flame atomic absorption spectrometer.

The above protocol was repeated using 80mM KBS instead of the 50mM KBS and both the supernatant and lysate were analysed. The percentage efflux was then calculated.

Cells treated with various concentrations of NS1619
The cells were incubated in RBS (200 µl/ well) for 4 hours, then washed four times with KBS (200 µl/well) as described above. Each column of the 12 columns of the 96-well plate containing the cells was then exposed to KBS (control), KBS+DMSO (control), 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100µM NS1619 (200 µl/ well) respectively, for 10-min. The supernatant was then transferred to a new 96-well plate and the cells lysed with 0.1%v/v Triton X-100 in KBS (200 µl/ well) for 18 hours. The volume of each well was made up to 300µl by adding 100µl KBS. Rubidium content was determined in both the supernatant and lysate samples with the standards of RbCl using the FAAS instrument. The percentage efflux of the various concentrations of NS1619 and the controls was then calculated.
Data Analysis

Rb⁺ efflux was calculated using the equation:

\[
\text{%Efflux} = \frac{[\text{Rb⁺}]_{\text{Extracellular}}}{[\text{Rb⁺}]_{\text{Extracellular}} + [\text{Rb⁺}]_{\text{Intracellular}}} \times 100
\]

Where: \([\text{Rb⁺}]_{\text{Extracellular}}\) = concentration of Rb⁺ in the supernatant plate

\([\text{Rb⁺}]_{\text{Intracellular}}\) = concentration of Rb⁺ in the lysate plate

Results were then presented as mean ±SEM and data analysed with Graphpad prism version 6.05 software using linear regression, parametric unpaired t-test, one-way analysis of variance (ANOVA) and dunnett t test with p<0.05 as level of significant.

3. Results

Rubidium Standard Calibration Curve

The calibration using standards of known Rb⁺ concentrations (10 - 100µM) in tubes done at the beginning and end of each of the analysis gave a linear relationship between the absorbance and concentration with correlation coefficient (R²) of 0.998 and 0.999 respectively and there was overlap between the points as shown in figure 1 (A).

The standard curve constructed from the mean absorbance of eight (8) data of both the calibrations (of Rb⁺ standards in the tubes) done at the beginning and the end of four (4) analysis gave a linear relationship between the absorbance and the concentration with correlation coefficient (R²) of 0.9993 (figure 1 (B)). Also, when standards of the known Rb⁺ concentrations (10 - 100µM) were put in a 96 well plate and analyzed, the Rb⁺ standard curve constructed from the data obtained also gave a linear relationship between the absorbance and the concentrations with correlation coefficient (R²) of 0.999 (figure 1 (B)).

Moreover, the rubidium standard calibration curve constructed from the data of the analysis of both the tubes and 96 well plate overlapped (figure 1 (B)) indicating precise calibration and internal validation. In addition, the statistical analysis showed no significant different between the initial and final calibration as well as between the Rb⁺ standards in the tubes and 96 well plate.
Although, known concentrations of $Rb^+$ standards were put in the 96 well-plate, the determined concentrations and absorbance of the standards in the plate from the analysis using flame atomic absorption spectrometer were same to those in the tubes as shown in table I.

Table I: The concentrations and absorbance of the standards of known $Rb^+$ concentrations in the tubes and the 96 well-plate obtained from the analysis using flame atomic absorption spectrometer. Data represented as mean absorbance and concentrations from replicate standards ± SD, n=8. The standard deviations (SD) of the absorbance of the standards (10, 30 and 40 µM) in the 96 well-plate were zero.

| TUBES | 96 WELL-PLATE |
|-------|----------------|
| Concentration of $Rb$ (µM) | Average absorbance ± SD (nm) | Concentration of $Rb$ ± SD (µm) | Average absorbance ± SD (nm) |
| 10 | 0.05 ± 0.01 | 9.50 ± 1.09 | 0.04 |
| 20 | 0.10 ± 0.01 | 21.16 ± 1.45 | 0.09 ± 0.01 |
| 30 | 0.15 ± 0.02 | 30.34 ± 1.09 | 0.13 |
| 40 | 0.20 ± 0.03 | 41.15 ± 0.88 | 0.18 |
| 50 | 0.25 ± 0.03 | 53.60 ± 1.85 | 0.23 ± 0.01 |
| 60 | 0.30 ± 0.03 | 64.58 ± 2.06 | 0.28 ± 0.01 |
| 70 | 0.35 ± 0.04 | 74.60 ± 2.34 | 0.32 ± 0.01 |
| 80 | 0.39 ± 0.04 | 84.78 ± 2.22 | 0.39 ± 0.01 |
| 90 | 0.44 ± 0.05 | 94.68 ± 2.65 | 0.41 ± 0.01 |
| 100 | 0.48 ± 0.05 | 104.84 ± 2.87 | 0.46 ± 0.01 |

Rubidium ($Rb^+$) Efflux Assay

_Loaded cells and washes (Un-stimulated cells)_

The HEK293 cells expressing the α subunit of BK cells after incubated in RBS (5.4mM of RbCl) for 4 hours, washed and lysed, the average concentrations of rubidium in the supernatant and the lysate obtained from the analysis using flame atomic absorption spectrometer were found to be 14.47µM and 56.24µM respectively as indicated in figure 2. In addition, the statistical analysis also showed there was significant difference between the supernatant and the lysate (p<0.0001).

![Figure 2: The $Rb^+$ concentrations of the supernatant and the lysate.](image-url)

HEK293 cells expressing α sub unit of BK channel were loaded with RBS, washed and then lysed with 0.1% Triton X-100. The $Rb^+$ content was determined using flame atomic absorption spectrometry as described under methods. Data shown as mean concentration ± standard error of mean, n = 96. The t-test analysis of the data gave p<0.0001 (****) as level of significant.
Cells treated with higher concentrations of KBS (Stimulated cells)

Cells treated with 50mM KBS
The data obtained from the analysis of the HEK293 loaded cells treated with 50mM or 5.4mM KBS for 10 minutes showed higher percentage of $\text{Rb}^+$ efflux in the former than in the latter. The average percentage efflux of $\text{Rb}^+$ of the 50mM and the 5.4mM KBS were 18.36% and 12.42% respectively as shown in figure 3. Statistical analysis showed significant different ($p<0.0001$).

![Figure 3: The efflux of rubidium due to exposure to 50mM KBS.](image)

Cells treated with 80mM KBS
The data obtained from the analysis of the HEK293 loaded cells treated with 80mM or 5.4mM KBS for 10 minutes showed higher percentage of $\text{Rb}^+$ efflux in the former than in the latter. The average percentage efflux of $\text{Rb}^+$ of the 80mM and the 5.4mM KBS treated cells were 22.37% and 20.60% respectively as shown in figure 4 (A) which statistically there was no different. However, when the % $\text{Rb}^+$ efflux of the treated cells with 80mM KBS was compared with the % $\text{Rb}^+$ efflux (12.42%) obtained from the 5.4mM KBS treated cells in figure 3, there was much higher difference in the % efflux as shown in figure 4 (B). This shows that the % efflux (20.60%) of the 5.4mM KBS was abnormally high.

![Figure 4: The efflux of rubidium due to exposure to 80mM KBS.](image)
Cells treated with various concentrations of NS1619 (Stimulated cells)
The HEK293 loaded cells after treatment with KBS, KBS+DMSO, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100µM NS1619 for 10 minutes gave average percentage rubidium efflux of 14.07%, 12.69%, 17.40%, 18.28%, 13.64%, 16.03%, 11.81%, 8.22%, 9.25%, 6.26%, 10.09% and 8.27% respectively. However only 0.1, 0.01 and 0.003µM NS1619 were higher than KBS, as shown in figure 5 below.

![Figure 5: The effect of NS1619 on Rb⁺ efflux](image)

**NS1619 Concentration**

HEK293 cells expressing a subunit of BK channel were loaded with RBS, washed, treated with various concentrations of NS1619 for 10 minutes and then lysed with 0.1% Triton X-100. The Rb⁺ content was determined using flame atomic absorption spectrometry as described under methods. Data shown as mean percentage efflux of rubidium ± standard error of mean, n = 8. Analysis using ANOVA comparing the various treatments gave p<0.0001 (****) as level of significant.

The Statistical analysis using ANOVA to compare the various treatments showed that there was significant different (p<0.0001). However, there was no significant different between KBS and KBS+DMSO.

4. Discussion
In this research, the rubidium standard curve constructed from the data obtained from the calibration of rubidium standards of known concentrations at the beginning and the end of each analysis (in the tubes) and that obtained from the analysis of the rubidium standards of known concentrations in the 96 well plate both yielded a linear relationship between the concentration and absorbance (figure 1) and showed similarity which indicated that there was consistency between each run of the analysis, there was also precise calibration and internal validation. Moreover, there was an absolute dependency between the absorbance and concentrations of the standards in the tubes and the 96 well plate; as the concentration increases, the absorbance also increases. This shows that the higher the concentration the more it absorbs light due to more molecule interaction and it is in accordance with Beer's law which showed that the absorbance is directly proportional to the concentration (A=єbc, where A is the absorbance, є is the molar absorptivity, b is the path length and c is the concentration). In addition, the instrument was able to analyze/calculate the exact concentrations of the standards in the 96 well-plate indicating that it is accurate and reliable.

The rubidium efflux assay using flame atomic absorption spectrometry in the analysis of BK potassium channel activity consists of two main parts: cell culture involving culturing of the cells, loading of cells with Rb⁺ followed by activation of the channels. The second part is the analysis using the instrument to determine the Rb⁺ concentration in the supernatant and the lysate.

In this research, rubidium was used as an indicator for potassium because it is permeable to the BK channel and they are in the same periodic group. It is however, easily excited and ionized in flames unlike potassium. The HEK 293 cells expressing the a subunit of BK channel were incubated with rubidium for four hours as previously determined by McKay et al. (2008) to be the optimal loading time (maximum time taken for Rb⁺ to attain the highest concentration possible in the cell with no significant increase in Rb⁺ entry above that time) and the cells were able to take up the rubidium indicated by more rubidium concentration in the lysate plate than in the supernatant (shown in figure 2). This shows that BK channels allowed movement of rubidium
into the cells and enters through the same route as potassium and they pass down their concentration gradient (from region of higher concentration to a region of lower concentration.

In addition, four washes of the cells after Rb⁺ loading was found to remove the extracellular Rb⁺ which was indicated by the higher level of Rb⁺ in the lysate. This however, was in accordance with previous study by McKay et al. (2008) where it was found that four washes were sufficient to effectively remove extracellular Rb⁺ with no significant removal on subsequent washes. Also, the outcome obtained despite the use of 5.4mM KBS for the four washes was an indication that the cells were not stimulated. This suggests that low concentration of KBS as 5.4mM will not result in depolarization of the cells or activation of the BK channels therefore has no effect in efflux of rubidium or potassium making it useful as a comparative measure in determining the stimulating effect of some compounds.

BK channel results in repolarisation of the membrane by allowing the efflux of potassium ion. The channel however, is activated in response to depolarization of the membrane which was achieved when the cells were treated with higher concentrations of potassium. The Rb⁺ loaded HEK293 cells expressing α sub unit of BK channel when treated with higher concentration of 50mM KBS for 10 minutes showed significantly higher Rb⁺ efflux than those treated with 5.4mM KBS giving a percentage increase of Rb⁺ efflux of 47.83% which indicated higher stimulation of BK channels and this was in accordance to study done by Scott et al. (2003) where it was found that increase in potassium chloride concentration led to increase in Rb⁺ efflux when HEK293 cells expressing the KCNQ2 (potassium) channel loaded with Rb⁺ were treated with 20, 30 and 50 mM KCl. Also, it was found that the Rb⁺ loaded cells expressing the KCNQ2 channel gave 400% increase in Rb⁺ efflux when treated with 50mM of KCl for 10 minutes whereas those expressing Kv1.1 or Kv1.4 gave 30% increase, suggesting that channel expression levels, conductance level and degree of inactivation (in case of Kv1.1 and Kv1.4) affect the extent of Rb⁺ efflux (Terstappen 1999; Scott et al. 2003)

However, the percentage efflux of rubidium as a result to treatment of the Rb⁺ loaded HEK293 cells expressing α sub unit of BK channel with 80mM KBS for 10 minutes showed higher Rb⁺ efflux than those treated with 5.4mM KBS giving a percentage increase of Rb⁺ efflux of 8.59% which was statistically not significant. Although, when the percentage efflux of the treated cells 80mM KBS was compared with the percentage efflux (12.42%) of that of the treated cells with 5.4mM KBS obtained initially (figure 3) it gave an increase in percentage efflux of 80.11% which was much higher than that of the 50mM KBS. This shows that the higher the concentration of potassium chloride the more it activates the BK channels leading to increase in efflux of potassium ions. However, the low increase (8.59%) in percentage efflux obtained was as a result of the high percentage efflux obtained from the cells treated with 5.4mM KBS (20.60%) as shown in figure 4 which could be as a result of failure to remove all the extracellular Rb⁺ during washing before the treatment with 5.4 mM KBS or the loaded Rb⁺ have naturally efflux out due to delay in washing since the Rb⁺ will move down its concentration gradient after attainment of equilibrium (that is after the optimal loading time) or the cells have been dislodged during washing leading to fewer cells in the lysate plate (McKay et al. 2008).

Nonetheless, NS1619 powder was dissolved in DMSO because of its insolubility in water. When the cells were treated with DMSO and 5.4mM KBS, there was no significant difference in the outcome relative to that of 5.4mM KBS alone, this shows that DMSO has no effect on the activation of the BK channels. In previous studies, NS1619 being a potent BK channel opener has been found to have a modulating effect in stimulating the BK channels resulting in efflux of Rb⁺. It has also been used in studies as a reference drug in determining the modulating effect of potassium channel openers and blockers (Kirby et al. 2013).

In this research, the average percentage efflux of rubidium obtained from different concentrations of NS1619 were compared with KBS and only 0.1, 0.01 and 0.003µμM NS1619 were higher than KBS which gave percentage increase in efflux of 13.98%, 29.95% and 23.69% respectively relative to 5.4mM KBS. This shows that 0.1, 0.01 and 0.003µμM NS1619 can lead to activation of BK channel with subsequently resulting in efflux of rubidium or potassium with 0.01µμM being the most effective dose, however, the activation is not consistent. Though, previous studies showed that NS1619 concentrations of 0.1, 1, 10 and 100µμM resulted in the activation of the BKα channels (McKay et al. 2008; Kirby et al. 2013). Also, the potency (EC40%) of NS1619 which is the concentration of NS1619 required to increase Rb⁺ efflux above baseline by 40% and its efficacy (Emax) which is the maximum increase in activated efflux, were found to be 0.32 ± 0.19 µM and 79 ± 9.8% respectively according to McKay et al. 2008 whereas 0.35 ± 0.1 µM and 61.7 ± 3.1% respectively according to Kirby et al. 2013.

However, according to Bentzen et al. (2014), NS1619 being the most widely used tool in the study of the functional effect of BK channels, has some drawbacks to its use which include: non established binding site,
poor potency and several off target effects such as inhibition of calcium channels. These have led to the generation of more selective and potent compounds for the study such as NS11021 (Bentzen et al. 2014).

Nevertheless, further research can be conducted to evaluate some molecules for BK channel activating potency using this method. Secondly, BK channel activation can further be analysed using higher concentrations of potassium chloride above 50mM to determine the precise concentrations required to stimulate the BK channels. Thirdly, the activating effect of NS1619 on the BK channels can be revisited since data generated from this research showed no consistency in its BK channel activation. In addition, the potassium channel opener NS11021 can be used in subsequent research in place of NS1619 to clarify its proposed potency. Also, BK channel expressing both α and β subunits can be used in the rubidium efflux since the β is thought to increase the functionality of the α subunit. Lastly, other ion channels with different cell lines can be studied using the flame atomic absorption spectrometry.

5. Conclusion
Rubidium efflux assay using flame atomic absorption spectrometry for the determination of potassium channel activity was confirmed to be accurate and reliable. The instrumental set up and calibration involve simple steps with less time consuming as it was capable of analysing samples of two 96 well plate in less than 2 hours. Also, the depolarisation effects of higher concentrations of potassium chloride and that of NS1619 on HEK293 cells expressing α sub unit of the BK channel were able to be determined which led to the activation of the channels with increase in rubidium efflux, although was not consistent with NS1619. This research shows the possibility of using flame atomic absorption spectrometry for rubidium efflux assay to test for compounds with BK channel activity which will aid in presenting more BK channel openers available for clinical trial and for future use in the treatment of some conditions.

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