PARA-AMINOBENZOIC ACID IN A POPULATION STUDY

USE OF PARA-AMINOBENZOIC ACID FOR VALIDATING 24-HOUR URINE COMPLETENESS IN A SODIUM-BLOOD PRESSURE POPULATION STUDY

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TITLE: Use of Para-aminobenzoic Acid for Validating 24-hour Urine Completeness in a Sodium-Blood Pressure Population Study

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ABSTRACT

Despite the fact that high sodium intake has been linked to high blood pressure and an increased risk for cardiovascular disease (CVD), sufficient and irrevocable evidence to support extremely low dietary sodium intake has been lacking. The best method used for the estimation of daily sodium intake is indirectly by using 24-hour urinary sodium excretion, combined with para-aminobenzoic acid (PABA), which is used for validating completeness of urinary collection. However, the PABA validation method is not favored among researchers and the validity of high-performance liquid chromatography (HPLC) analysis for PABA has not been evaluated in a large-scale study. This study validated an improved PABA HPLC method and applied it in the sub-set of the Prospective Urban Rural Epidemiology (PURE) – 24-hour Urinary Excretion (24USE) Study for the examination of the sodium-blood pressure association. In addition, participants' self-reported assessment of urinary collection completeness was compared to the PABA method.

The HPLC analysis method for measuring PABA levels was downscaled and the reaction time lengthened to achieve higher through-put and reaction yield. By applying the optimized PABA method for evaluating urinary completeness, 612 of 681 participants' samples contained 70%-110% PABA recovery and were extracted from the PURE24USE Study for further analysis. The average adjusted sodium excretion or intake was 3,673 ± 1,637 mg/day. The participants' self-reports predict urinary collection completeness as

measured by PABA with a sensitivity of 76.9% (95% CI: 74.4%-79.6%) and specificity of 31.7% (95% CI: 27.0%-36.4%). This moderate agreement suggests that PABA still has to be considered the gold standard, until further convincing evidence is available that self-report is more accurate than PABA.

ACKNOWLEDGEMENT

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List of Abbreviations

24USE	24-hour Urinary Sodium Excretion Survey
ANOVA	analysis of variance
BMI	body mass index
CCHS	Canadian Community Health Survey
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CV	coefficient of variation
CVD	cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
FFQ	food frequency questionnaire
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
IOM	Institution of Medicine
LOD	limit of detection
LOQ	limit of quantification
NaOH	sodium hydroxide
NHANES	National Health and Nutrition Examination Survey
NPV	negative predictive value
PAAB	para-acetamidobenzoic acid
PABA	para-aminobenzoic acid
PAH	para-aminohippuric acid
PAHA	para-acetamidohippuric acid
PPV	positive predictive value
PURE	Prospective Urban Rural Epidemiology Study
QC	quality control
SD	standard deviation
SE	standard error

Se	sensitivity
Spe	specificity
TOHP	Trials of Hypertension Prevention

Declaration of Academic Achievement

Participant enrollment, data collection, sodium, and urine volume measurement had been performed by the PURE24USE working group. I was responsible for the urine volume validation, PABA method validation and PABA measurement. Every table, figure and analysis was made by me.

I. STUDY OVERVIEW AND OBJECTIVE

A. Study Overview

Cardiovascular disease (CVD) is the leading cause of death in Canada accounting for 29% of all deaths [1]. One of the efforts to address the high prevalence of CVD among Canadians is to reduce the sodium intake to less than 2,300 mg per day [2], based on the finding that excessive sodium intake is associated with high blood pressure, a major risk factor for CVD [3]. However, the impact of low sodium intake (<2,300 mg/day) on CVD outcomes is controversial, with recent cohort studies showing that lowering sodium intake to these levels can be associated with increased CVD and mortality [4]. Recently, an Institute of Medicine (IOM) expert panel convened to study the evidence and concluded that there was insufficient data to lower sodium intake to less than 2,300 mg/day [5]. Importantly, the report called for more studies of healthy populations with a broad range of sodium intake to better help characterize an optimum level of intake.

Numerous existing studies have examined the relationship between sodium and CVD. However, until recently most of these studies have used dietary assessment methods (e.g., food frequency questionnaire [FFQ]) to assess sodium intake. Although these methods are convenient, have low cost, and are suited for assessing long term dietary intake in large epidemiology studies, they are prone to inaccurate recall of dietary intake and do not account for salt added at the table or during cooking. Therefore, urinary

collection is still the most accurate method to measure sodium intake. At present, 24-hour urine collection is the 'gold standard' for estimating sodium intake [6]. Clear advantages of 24-hour urinary measures are the objective nature of the measurement, and the ability to apply across diverse populations in a standardized manner. However, a major limitation of 24-hour urinary measures is that they underestimate true levels of intake when the urine collection is incomplete.

In the past, urinary creatinine was used to check for completeness of urine collection, but the high variability associated with its excretion makes identifying under collection difficult. More recently, creatinine has been replaced by para-aminobenzoic acid (PABA), an intake marker that is ingested with the three main meals on the day of urine collection and is rapidly absorbed and almost completely excreted within 24 hours [7]. It is generally safe and exists naturally in many food items.

Since the development and implementation of PABA as an intake marker, methods for analysis have undergone major modifications due to the advancements of technology. The newer high performance liquid chromatography (HPLC) analysis method [8] has fewer interferences from other drugs and results in more accurate measurement of PABA compared to the original colorimetric method [7]. However, the validity of this HPLC method has not been completely evaluated in a large epidemiological study. Many studies that used the HPLC method still adopted the complete urine collection criterion based on the old colorimetric method. Incomplete

urine collections are often included in further analysis based on an assumed and as yet poorly examined association between excreted PABA and sodium. Furthermore, the extra cost associated with the PABA method can render this method less desirable, and thus limit its application. The principal goals of this thesis are to investigate and validate the use of PABA as an intake marker for assessing completeness of 24-hour urine collection in a subset of the Prospective Urban Rural Epidemiology – 24 Urinary Sodium Excretion (PURE24USE) study, to determine if the completeness of urine samples can be assessed using participant responses from an accompanying questionnaire, and to compare the levels of sodium excretion in 24-hour urine collections, which have been checked for completeness using the PABA method versus self-reporting method.

B. Objectives

1. The published HPLC method for PABA analysis has uncertain analytical detail and will be modified and validated as a reference procedure for the validation of the completeness of a 24-hour urine collection.
2. The validated method for PABA measurement and completeness of the 24-hour urine collections will be applied to the examination of the link between sodium intake and blood pressure in this sub-set of the PURE24USE Study.
3. Statistics for the agreement of self-report with PABA as reference method to assess completeness of 24-hour urine collections will be calculated. The

statistical measures will be interpreted regarding the validity of self-report and inform decisions on the necessity of PABA analysis in future studies.

II. LITERATURE REVIEW

A. Sodium, Blood Pressure and Cardiovascular Diseases

Salting of food is one of the most common flavoring and preservation techniques, and such practice can be traced back 5,000-10,000 years [9]. In recent years however, the awareness of the negative impacts of high sodium intake on cardiovascular health has been increasing. High sodium intake is found to associate with high blood pressure, which then influences the prognosis of CVD. About 19% of the Canadian population is affected by high blood pressure and CVD is one of the major leading causes of death in the past decades [9]. In addition, CVD is the second largest contributor to the health cost in Canada. Lowering the dietary sodium intake at the population level could be one solution for the current CVD status and the related economic burden. Health Canada has recommended lowering the daily sodium intake to less than 2,300 mg, as compared to the current average Canadian intake of 3,400 mg [10]. Although the recommendation to lower sodium intake has existed for decades in several countries, the level of optimal sodium intake remains uncertain and the effect of low sodium intake on blood pressure and CVD has remained inconclusive.

II. LITERATURE REVIEW

Sodium and Blood Pressure

Hypertension is a multifactorial disease caused by the combined effect of genetic influences and external environment. Sodium is thought to be the major environmental factor that influences blood pressure. Before the 20th century high blood pressure was thought to be caused by protein intoxication [11]. Although this hypothesis was soon negated by Ambard and Beaujard, who found that blood pressure was not influenced by changes in protein content in diet [12], the exact pathophysiology of hypertension remained elusive. At the time, numerous methods were proposed to treat hypertension. Diet modification, more precisely, salt restriction was found to be an effective treatment for hypertension; however, chloride was first thought to be the main effector at the time.

The association between sodium and high blood pressure was initially derived from studies that used diet modification to treat hypertension [12-16]. Ambard and Beaujard treated severely hypertensive patients with a special diet regimen and found that the blood pressure of hypertensive patients rose and fell as a response to changes in salt content of their diets [12]. They attributed the blood pressure change to chloride [12], and that view was shared by Allen and Sherrill [13] and Kempner [14, 15]. Allen and Sherrill believed that hypertension was caused by an inability of the kidney to excrete chloride; therefore, hypertension could be treated by avoiding salt in the diet [13]. In their study, hypertensive patients were admitted to hospital and received diet treatment for about two weeks and were followed for up to five years. About 75% of the patients benefited from

the salt restricted diet [13]; however, the exact definition of benefit was not specified. Because this study was uncontrolled and the compliance with the diet regimen was not documented, the validity of the study was questionable. Kempner also believed that hypertension could be treated with diet and postulated that hypertension was caused by "abnormal substances" released into the blood by overworked kidneys [14, 15]. Therefore, by reducing salt, protein and fat in the diet, the production of these "abnormal substances" could supposedly be reduced. Of the 500 patients receiving his special diet containing rice, fruit and sugar, 25% of the patients had restored normal blood pressure and 67% lowered their blood pressure by more than 20 mmHg [15]. The success of Kempner's rice diet provoked further examination of the subject. Grollman *et al* found that the striking change in blood pressure was mostly due to lower amounts of salt in the rice diet rather than protein or fat as proposed by Kempner [16]. Furthermore, sodium was found to cause the blood pressure lowering effect rather than chloride [17]. Dole *et al* examined the sodium effect of Kempner's rice in six hypertensive in-patients observed continuously for six months. They found that the blood pressure lowering effect of the rice diet could only be reversed with addition of sodium in the diet but not with chloride. Despite flaws in study design and an ongoing debate over the role of chloride [18], this early work provided important insights into the relationship of sodium and hypertension.

Several population studies revealed interesting observations on the relationship between sodium and blood pressure [19-22]. Dahl measured the salt intake among five

geographically distinctive populations with an average sodium intake ranging from 1,600 – 10,300 mg/day and found it to have a significant positive linear relationship with the prevalence of hypertension within the population (Figure 1) [19]. The studies conducted by Gleibermann [20] and Froment *et al* [21] also supported Dahl's observation. Gleibermann examined the blood pressure of men aged 50-55 years and their salt intake within 27 populations and found that blood pressure rises with sodium intake [20]. Froment *et al* evaluated the association between systolic blood pressure and 24-hour urinary sodium excretion of 50-year-old men among 28 populations using published data and found a significant positive relationship (Figure 2) [21]. Although the results shown by these studies were intriguing, flaws in study designs decreased the value of these studies. The study by Dahl was based only on five populations. In the study conducted by Gleibermann, the quality of the study was greatly affected by inconsistency in blood pressure measurements, subject selection and urine collection. In addition, the positive significant relationship found by Froment *et al* became trivial when data from nine isolated populations with low sodium intake (average sodium intake less than 790 mg/day) were excluded (Figure 2). The validity of the above mentioned studies also suffered from confounding factors. Possible confounders of blood pressure such as weight, potassium intake and alcohol consumption were not included. Furthermore, these multi-population studies were especially susceptible to confounders due to the socioeconomic, geographic and environmental factors that can affect blood pressure. More standardized studies were

therefore required to convincingly demonstrate the relationship between sodium and blood pressure.

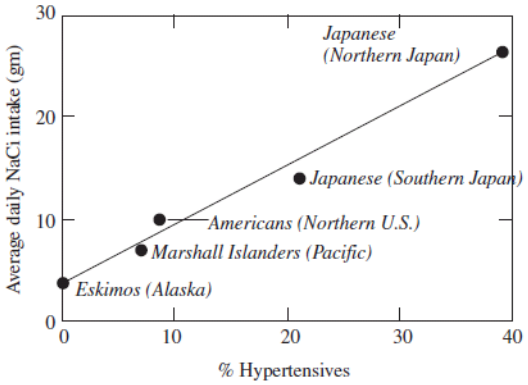

Figure 1: Prevalence of hypertension was positively associated with sodium intake among five geographically distinctive populations [19].

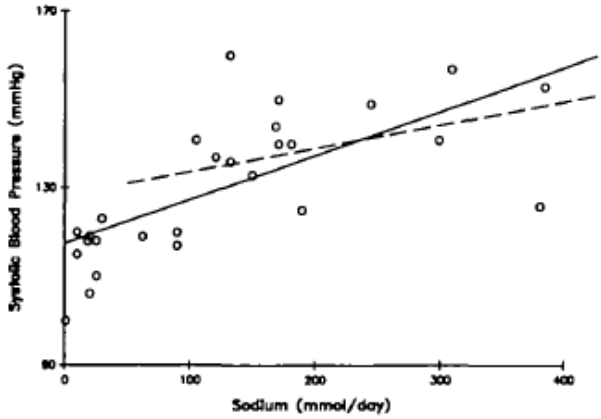

Figure 2: Association between sodium and blood pressure became non-significant when 9 isolated, low sodium intake populations were excluded [21].

II. LITERATURE REVIEW

Intersalt, begun in 1981, enrolled 10,079 participants from 32 countries in a total of 52 centers and attempted to correct the drawbacks in the previous studies [22]. It was the first standardized and systematic study and it included confounders in the analysis. The relationship between median 24-hour urinary sodium and blood pressure was examined and a significant association was found across 52 centers ($p < 0.001$), however, the association was weakened after adjustment for age, sex, body mass index (BMI) and alcohol consumption ($p < 0.01$). Four non-industrialized populations from Brazil (Yanomamo and Xingu), Papua New Guinea and Kenya were also included in the study. Participants from these four populations had low average sodium intake ranging from 5 – 1,180 mg/day and low systolic and diastolic blood pressure between 95/61 – 110/68 mmHg. By excluding these four populations that were very different from the rest, the previously established positive association between sodium excretion and blood pressure disappeared and a negative association was seen (Figure 3) [22]. This greatly impacted the tenability of a positive association between sodium and blood pressure.

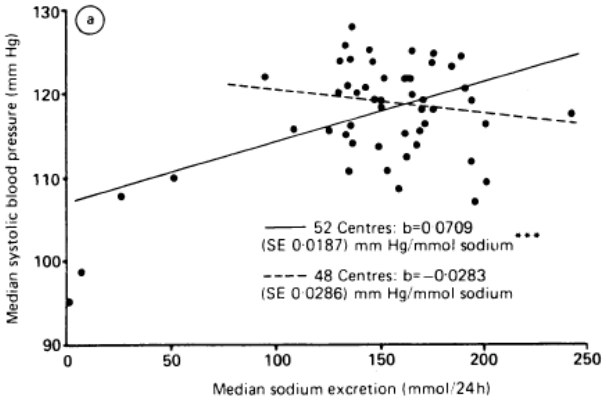

Figure 3: The positive association between sodium and systolic blood pressure in INTERSALT became negative once four isolated populations were excluded from the analysis [22].

The association between sodium and blood pressure within populations was also assessed in several other studies [3, 23-25]. Smith *et al* examined 7,300 participants in Scotland and found the association to be weak within this population [23]. In Intersalt, the significant positive relationship between sodium excretion and blood pressure in individuals was strengthened after correcting for regression dilution bias in the day-to-day individual sodium excretion [3]. Within population studies usually suffered from insufficient statistical power due to variation in daily sodium excretion which does not represent the habitual sodium intake. This random error in the x-variable introduces not only imprecision, but also regression dilution bias, an underestimation of the slope [26]. This counter-intuitive effect, a bias caused by noise, is due to the asymmetry of simple linear regression, which takes only error in the y variable into account. Regression

dilution is a consequence of regression to the mean: Values at the extremes will likely be measured as less extreme the second time.

In addition to the population studies, intervention studies demonstrated the blood pressure lowering effect of reduced dietary sodium in hypertensive patients [27-30]. By providing 30 days of the DASH (Dietary Approaches to Stop Hypertension) diet containing low sodium, Sacks *et al* successfully lowered blood pressure in both normotensive and hypertensive participants [28]. A decrease in sodium intake from 3,300 mg to 1,500 mg translated into a fall in systolic blood pressure by 11.5 mmHg for hypertensive participants and a 7.1 mmHg decrease in normotensive participants [28]. Nevertheless, the blood pressure lowering effect could not be entirely attributed to sodium reduction, since the DASH diet itself is low in fat and rich in fruit, both of which provide anti-hypertensive effects [28, 30]. The study also found that the blood pressure lowering effect of reduced sodium intake was more effective in African Americans and women. Another intervention study, Trials of Hypertension Prevention (TOHP), was carried out in 1987 [27]. By lowering daily sodium intake by 1,000 mg from the baseline of 3,600 mg, the blood pressure of healthy individuals was lowered by 1.7/0.9 mmHg ($p < 0.01$) [27]. A sodium restricted diet may be an effective method for treating hypertension. This may be less applicable to a normotensive population, however, because the sacrifice made on food taste is not cost-effective and the benefit is not imminent.

II. LITERATURE REVIEW

Sodium and Risk for Cardiovascular Diseases

Several studies showed that blood pressure of hypertensive patients could be reduced by lowering sodium intake [28-30]. Since hypertension is an important predictor for CVD, lowering sodium intake should in theory decrease the risk of CVD through its antihypertensive effect. Based on this assumption, several salt reduction initiatives have appeared. However, to date, conflicting results have been reported by studies that attempted to examine the association between extremely low sodium intake and CVD outcomes [4, 31-38].

Lowering sodium intake was found to be associated with lower incidence of CVD and mortality in some studies including those conducted by He *et al* [31], Tuomilehto *et al* [32], and Cook *et al* [35]. He *et al* found that in overweight adults, risk for CVD was strongly and significantly associated with sodium intake. However, this association was not found to be significant in individuals with normal weight [31]. Tuomilehto *et al* also found a similar association in a random sample of 2,436 Finnish participants aged 40-59 years. Increase of 24-hour sodium excretion by 2,400 mg/day was found to associate with an increase of 51% in CVD and 22% in all-cause mortality [32]. In a follow-up study of the TOHP, the long-term effect of sodium reduction on CVD was demonstrated by Cook *et al* [35], where pre-hypertensive participants were randomized into control and intervention groups. By lowering sodium intake by 25-30% from the original 4,000 mg/day, the blood pressure of the intervention group decreased by 1.7/0.9 mmHg after 18

months. After 10-15 years follow-up, the intervention group had 25% fewer CVD incidents after correction for confounders [35]. However, the long-term follow-up involved only 77% of the original participants, and only questionnaires were used to evaluate the result of the intervention instead of direct physical examinations. Nevertheless, the above mentioned cohort studies clearly showed a benefit of lowering sodium intake on cardiovascular health.

On the other hand, lowering sodium intake was also shown to be associated with increased risk for CVD and mortality in other studies [4, 33, 36, 37]. Cohen *et al* analyzed data obtained from the National Health and Nutrition Examination Survey (NHANES) II and found that a lower sodium intake (< 2,300 mg/day) was associated with increased risk of CVD mortality [36]. A similar conclusion was drawn from the NHANES III survey by Cohen *et al* [37]. Compared with participants with sodium intake over 4,000 mg/day, participants with sodium intake less than 2,000 mg/day had a significantly higher risk of CVD mortality ($p < 0.05$) after an average follow-up period of 8.7 years. Nevertheless, people in the higher sodium intake group were mostly male and younger and therefore tended to be healthier and should have had lower CVD mortality in theory. Stolarz-Skrzypek *et al* published their findings on the adverse effect of low sodium intake on risk of CVD. It was a long term observational study based on 3,681 normotensive participants with average 7.9 years follow-up. It was found that CVD related mortality had an inverse relationship with 24-hour urinary sodium [4]. This study

was faced with skepticism [39, 40]. Accurate measurements of daily sodium levels could not be guaranteed because under-collection of 24-hour urine was suspected. The study participants were provided with only a single 2.5 L container, which may not be sufficient to contain all 24-hour urine. In addition, confounder effects were not taken into consideration, the participants with lowest sodium intake had lower level of education, which was a factor for CVD risk as well [41].

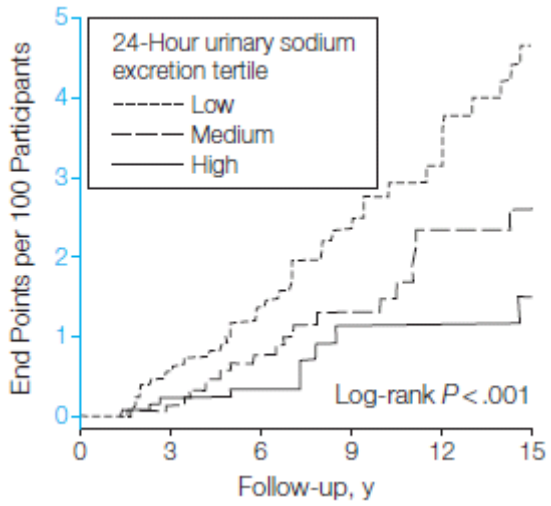

Figure 4: Low sodium intake was found to associate with higher CVD risks [4].

Another study that disputed the sodium reduction practice was performed by O'Donnell *et al* [33]. Unlike previous studies, that clinical trial found that the relationship between sodium intake and the risk of CVD was not linear, but rather had a J-shaped

association (Figure 5), with the sodium intake ranging between 4,000-6,000 mg/day having the lowest adverse health outcome. This retrospective cohort study was challenged for its external validity because morning spot urine adjusted with the Kawasaki formula was used for the estimation of daily sodium intake [42]. Spot urine was considered imprecise and unreliable for the estimation of daily sodium intake, especially when a healthy population was used for the validation of the spot urine when the study participants were mostly sick and on various medications including loop diuretics, which greatly affected the sodium excretion pattern of the participants [42].

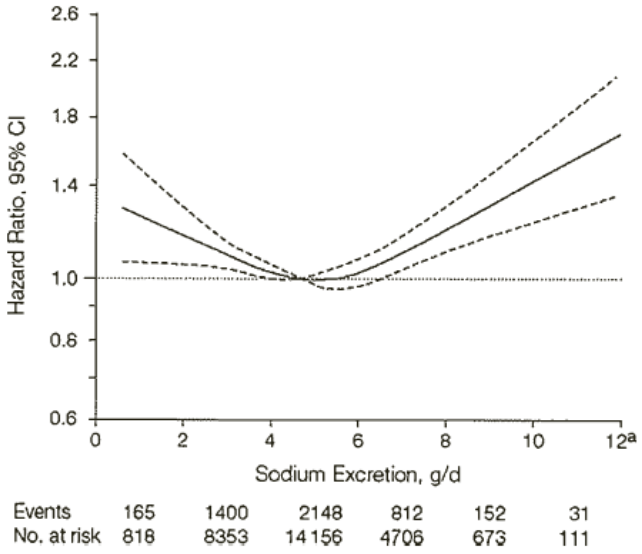

Figure 5: Sodium intake ranging between 4,000-6,000 mg/day was found to associate with lowest risk for CVD [33].

The above section described three conflicting views on the association between low sodium intake and CVD outcome. The IOM attempted to elucidate this confusion.

However, after the data had been gathered from existing studies, the IOM concluded that at that moment there was insufficient evidence to support lowering sodium intake to less than 2,300 mg/day [5]. In conclusion, more research is required to settle the dispute on the association between low sodium intake and CVD outcome.

Sodium Intake Measurement

One factor that hindered the understanding of relations between sodium intake and CVD was the inconsistency of the study designs to evaluate effects of low sodium intake. No standardized method was used for measuring sodium intake across the existing studies, and the daily amount of sodium considered as high intake, used as a comparator for the effect of low sodium levels, varied greatly from 2,700 mg/day to 10,000 mg/day. Currently, the available methods used for quantifying sodium intake include interview, questionnaires or urinary sodium measurement as surrogates for daily sodium intake.

Dietary Sodium Intake Assessment

Instruments used for assessing dietary sodium intake include 24-hour dietary recall and food frequency questionnaire (FFQ). The 24-hour dietary recall, used in the NHANES study, is an interview survey that records all the food or drink items consumed during the past 24 hours [43]. This method has high compliance among participants and can be readily employed in large population studies because the participants do not require high literacy. However, this method often suffers from under-estimation of the true sodium intake level because some food items may not be included or are labeled

with lower sodium content than the true value. In addition, 24-hour dietary recall is often associated with reporting bias and lack of habitual intake information. These can be improved or even avoided when the interviews are conducted by specially trained personnel; hence increased accuracy is achieved at the expense of increased cost and time. FFQ, on the other hand, is the most convenient and a relatively inexpensive method for evaluating sodium intake [44]. It uses a questionnaire that collects information on how often and how much of each food item is consumed. Self-administration is possible for FFQ and evaluation of the habitual sodium intake is possible. However, FFQ is susceptible to reporting bias and, similar to food recall, under-estimation of the sodium level.

Dietary Sodium Intake Assessment Using Urinary Sodium as Surrogate

The current reference standard for assessing daily sodium intake is by using 24-hour urinary sodium excretion as a surrogate for intake since about 90% of sodium is excreted in urine. Dahl *et al* first proposed the use of 24-hour urinary sodium as a surrogate [19]. Large variation existed for inter-person and intra-person sodium excretion. Multiple 24-hour urine samples were proposed to compensate for this variation, and Luft *et al* found that the association between actual sodium intake and the average 24-hour urinary sodium over nine days had a correlation coefficient of 0.75, with the sodium intake being constantly underestimated [45]. Despite large between-day variation in sodium intake, a single 24-hour urine is most often employed. However, collecting 24-

hour urine is cumbersome for study participants and complete compliance is not guaranteed. Incomplete collection can occur due to missing urine sample, forgetfulness and misplacement of urine sample. These can affect the validity of the study. It is therefore important to account for the loss of collection.

Because collecting 24-hour urine is tiresome and creates a burden to the study participants, overnight urine and spot urine were proposed to replace 24 hour urine. Overnight urine collection has high correlation with the 24-hour urine with regression coefficients ranging from 0.75-0.94 [46-48]. However, the intra- and inter-person coefficients of variation are higher than those of 24-hour collection, making overnight collection less favorable for the purpose. Casual spot urine was also proposed for evaluating daily sodium intake, using excreted creatinine as reference; however, there was substantial variation in regression coefficients between 24-hour urine and spot urine when different methods were used (0.28-0.67) [49]. In addition, Kawasaki *et al* found that the sodium in a morning fasting spot urine sample did not reflect the sodium consumed over a 24-hour period and that multiple morning fasting spot urines would be more reliable than a single spot urine sample [50]. However, in the study by Kawasaki *et al* no precaution was taken to ensure their experimental protocol was followed or to ensure the completeness of 24-hour urine collection. The correlation coefficient could be improved to 0.73 using an adjustment equation involving urinary creatinine excretion [50]. However, this equation was developed based on a Japanese population with an

average sodium intake of 5,000 mg/day, which is much higher than the average sodium intake within the Canadian population of 3,200 mg/day. In short, problems regarding reliability or validity have prevented the wide spread use of the more convenient casual urine sample to replace 24-hour urine for the determination of sodium consumption.

Para-aminobenzoic Acid Analysis

As mentioned above, the evaluation of sodium intake using 24-hour urinary sodium as surrogate often faced the under-collection problem. Two main markers have emerged over the years for the determination of the completeness of 24-hour urine collection, the endogenous biomarker creatinine and the exogenous marker para-aminobenzoic acid (PABA). Urinary creatinine was first applied for this purpose. It is one of the most commonly used biomarkers because it does not subject the participants to an additional drug and there is no extra cost associated since it is endogenous. The excretion of creatinine is highly correlated to body mass [51]; therefore, the amount of creatinine excreted by an individual should, in theory, stay constant over the 24-hour collection period. Incomplete collection can be identified by comparing the measured urinary creatinine value with reference range values. However, creatinine excretion is highly variable and affected by exercise [52], meat consumption [53], age and gender [54] etc. Therefore, creatinine is unreliable for the determination of the completeness of urine collection due to these variations. PABA on the other hand, has become the main reference marker used for this purpose.

II. LITERATURE REVIEW

PABA was first introduced as an excretion marker in 1983 [7]. Bingham and Cummings demonstrated that 99% of PABA was excreted within 6 hours after ingesting an 80 mg dose of PABA. They also found that 93% of ingested PABA was recovered on average in 24 hour urine when a single 80 mg PABA pill was taken with each main meal. A sample containing over 85% of ingested PABA was considered a complete urine collection. The results found by Bingham and Cummings were considered as highly reliable, because the participants were confined in a room without a washroom during the urine collecting period [7]. However, PABA is often ignored in favour of other less reliable methods, because the added logistic effort of administering pills is expensive and time consuming [55, 56].

PABA is a precursor to the formation of folate in plants and bacteria. The absorption of PABA occurs in the small intestine through passive diffusion. PABA is metabolized in the liver to para-aminohippuric acid (PAH), para-acetamidobenzoic acid (PAAB) and para-acetamidohippuric acid (PAHA) (Figure 6) through phase II conjugation pathways [57] before being excreted in urine. Two main methods have been adopted by researchers for the determination of PABA in urine, colorimetric [7] and high-performance liquid chromatography (HPLC) methods [8]. For colorimetric PABA analysis, Bingham and Cummings [7] adapted the modified Bratton and Marshall reaction [58] by Tetlow *et al* [59] for the alkaline hydrolysis and detection of PABA metabolites. Basically, PAHA and PAAB are deacetylated to PAH and PABA

respectively through alkaline hydrolysis, and then PAH and PABA are coupled as diazonium salts with a reagent to form azo dyes through the Bratton and Marshall reaction. By measuring the amount of azo dye produced, the amount of PABA in the urine can be inferred. By the nature of the Bratton and Marshall reaction [58] however, other aromatic amines can engage in the detection through this process. Therefore, amine-containing drugs such as sulfonamides, folic acid, paracetamol, phenacetin and furosemide can also be detected along with PABA. Bingham and Cummings tried to compensate for this by measuring the amount of PABA both before and after the day of PABA ingestion [7]. However, no significant change was observed in the PABA recovery after the correction using the urine that contained no given PABA. Nevertheless, because of the possible interference from aromatic amine drugs, over-estimation of PABA excretion can occur and therefore, an incomplete urine sample can be misclassified as complete. This can impact the result of epidemiological studies. Therefore, when using PABA as indicator for determination of 24-hour urine collection, participants who are on sulfonamide-based drugs should be excluded, if a colorimetric method is used.

II. LITERATURE REVIEW

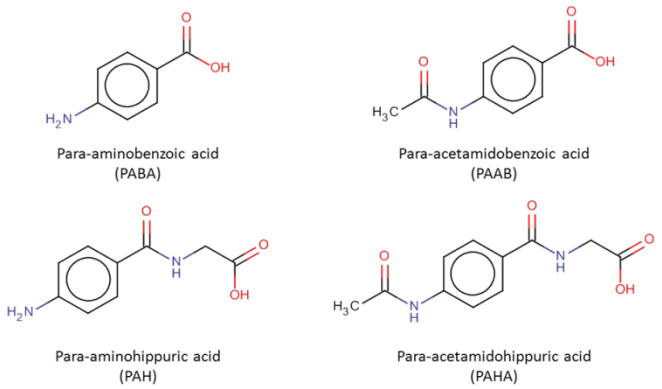

Figure 6: Chemical structure of PABA and metabolites

To improve the specificity of the PABA analysis Jakobsen *et al* reported the use of HPLC [8]. This method measures the amount of PABA in the urine after converting the metabolites to PABA with alkaline hydrolysis at 121°C for 90 minutes. This method is simple and highly specific and has become the main method for analyzing PABA recovery in 24-hour urine. Jakobsen *et al* also set the criteria for complete recovery at 78% of ingested PABA (n = 56) [8]. The difference between the complete collection criteria for Jakobsen *et al* and Bingham & Cummings could be caused by the fact that Jakobsen *et al* included subjects with a wider age group (20-80 of age) [8] compared to those of Bingham & Cummings (22-55 of age) [7]. Older age was found to associate with delayed PABA excretion due to decrease in kidney function [8, 60].

An accurate measurement of urine volume is necessary to establish an accurate PABA recovery result. However, urine volume measurement has not often been studied due to the simplicity of the task. Frequently used methods employ a graduated cylinder or

weighing. Measuring volume with a graduated cylinder is the standard method; however, pouring urine into different graduated cylinders is required rendering it an unpleasant task. Weighing, on the other hand, is simple, direct and precise [61, 62]. Weighing is especially useful when measuring large volumes. However, to accurately determine the volume, the laboratory balance must be of high weighing capacity and resolution, which can be expensive and not readily available in every lab. Furthermore, the reference urine specific gravity value often differs from the true value, and this may create a bias in the final PABA recovery result. Another method frequently used in the hospital setting requires a standardized container and a ruler with markings calibrated in volume units. For this study, the ruler volume measuring method was adopted and the precision and accuracy of this method was investigated.

III. EXPERIMENTAL

A. Source of Data and Study Sample

All data and urine samples in this study were obtained from the PURE24USE study, which aims to evaluate the current sodium intake status of residents from four cities in Canada. The study enrolled the PURE study participants who were attending their 3-year follow-up visits. The PURE study is an ongoing prospective cohort study that evaluates the effect of societal influences on lifestyle behavior, CVD risk and the incidence of chronic non-communicable diseases. 10,293 participants from 81 urban and rural communities in four sentinel sites (Hamilton, Ottawa, Quebec and Vancouver), have

been recruited in Canada [63]. The PURE24USE study participants recruitment was stratified by community, where 200 participants were consecutively sampled within each urban and rural area from four sentinel sites to make up a total of 1,600 participants. The major exclusion criteria were pregnancy or lactation; allergic to hair dye, sunscreen or vitamins; currently on sulfonamide medication; diagnosed with a debilitating disease with reduced survival; chronic illness that restricts food choice; and missing data from the PURE study. Participants were enrolled between February 2012 and December 2013 and informed consent was obtained from the final number of 1,700 PURE24USE study participants. Basic demographic information (age, gender, weight, etc.) was recorded.

Urine Collection

PURE24USE participants were asked to collect one 24-hour urine sample. They were provided with two 4-liter containers, three 80 mg PABA pills (for participants aged below 66 only), a detailed urine collection instruction and a urine collection sheet. On the day of 24-hour urine collection, participants were asked to take one PABA pill with each of their main meal (8am, 12pm, and 6pm). On the urine collection sheet, they were asked to record each PABA ingestion time, start and end time of their 24-hour urine collection. Two questions were also included for them to self-evaluate the completeness of urine collection: Question 1 “During the 24-hour collection period, did you always void into the urine collection container”; and Question 2 “Is any urine missing from the collection for any other reason.” Of the 1,700 participants from PURE24USE, 1,275 took PABA

during 24-hour urine collection and 681 took PABA at the anticipated time. Urine samples from these 681 participants were used during this study. Urine samples from those who did not take PABA (aged over 66) were used as negative controls.

When the urine samples were received from the participants at each center, the volume of the urine was measured and recorded. Samples were pipetted into two 2 ml storage vials. The aliquots were frozen before shipping on dry ice to the central laboratory in Hamilton within one month. In the central laboratory, aliquots were stored in a -70℃ nitrogen tank until the day of analysis, when they were thawed to room temperature and centrifuged for 2 minutes at 2,500 rpm (ALC Refrigerated Centrifuge PM140R).

Urine Volume Measurement Validation

The 24-hour urine samples were measured inside the 4-liter containers provided to the study participants. Using a ruler with volume marks on the side, the volume of the urine was read and recorded. The accuracy of the volume reading by ruler was assessed by a simulation study of volume measurement using water. The volume of a random amount of water was added to a 500 ml graduated cylinder, and the volume was recorded. It was later poured into the 4-liter standard issue container and the volume was measured using the ruler. More random amounts of water were added to the same container and measured using both methods until the 4-liter mark was nearly reached. This process was repeated four times. A total of 42 measurements were obtained. Because urine collections

were measured by different laboratory technicians at each center, the inter-person variation was also investigated. Four laboratory technicians were asked to assess the volume of a random amount of water in the 4-liter container using the ruler. These volumes were then compared with the reference volume measured using a graduated cylinder. Statistical comparisons were performed using linear regression and the Altman Bland comparison test in the Microsoft Excel 2007 add-on Analyze-It. The slope and the Pearson correlation coefficient were used to evaluate the association between two methods. Bias was also reported to estimate the agreement between the two methods.

B. Para-aminobenzoic Acid

Chemicals and Reagents

Para-aminobenzoic acid (PABA) and two of its metabolites, para-aminohippuric acid (PAH) and para-acetamidobenzoic acid (PAAB), were purchased from Sigma. The third metabolite, para-acetamidohippuric acid (PAHA), was synthesized from 0.4 ml of acetic anhydride (BDH) and 1.0 ml of 1.0 mg/ml aqueous PAH. This mixture was heated for 3 hours at 30℃ with intermittent shaking. The purity of the PAHA was confirmed using HPLC. HPLC grade methanol and acetonitrile were purchased from Fisher Scientific.

High-Performance Liquid Chromatography

The HPLC system used was from Varian and equipped with a quaternary pump (Prostar module 240), auto-sampler (Prostar module 410), column oven (Prostar module

510) and a deuterium UV-Vis detector (Prostar module 325). A HiChrom ultrasphere ODS reversed-phase analytical column (150 X 4.6 mm; 5 um) was used at 30℃ and the autosampler cooler was set to 4℃. The injection volume was 25 µl. The detector wavelength was set to 290 nm. The data acquisition and handling were performed by Varian Star Chromatography Workstation Version 6.41.

For the quantification of PABA concentration, isocratic elution at 1.0 ml/min was used. The mobile phase consisted of 315 ml of 0.1 M citric acid (Sigma), 135 ml of 0.2 M sodium phosphate dibasic (EMD) and 41.5 ml of acetonitrile (8.4%). The retention time for PABA was at 5.0 minutes.

For the simultaneous quantification of PABA and PAH, the flow rate was set to 0.9 ml/min for 4.5 minutes, 2.0 ml/min for 4.5 – 7.1 minutes and back to 0.9 ml/min from 7.1 – 7.3 minutes. The mobile phase consisted of 315 ml of 0.1 M citric acid, 135 ml of 0.2 M sodium phosphate dibasic and 23.5 ml of acetonitrile (5%). The retention times for PABA and PAH were 6.2 minutes, and 3.7 minutes respectively.

Calibrators Preparation

A PABA stock solution of 1 mg/ml was prepared in deionized water, following serial dilution to make up diluted PABA solution with concentrations ranging from 250 µg/ml – 10 µg/ml. Blank urine was prepared by pooling urine samples of patients over 64 years of age. The calibrators were then prepared by mixing 20 µl diluted PABA solution, 20 µl of 8 M sodium hydroxide (NaOH) (BDH), 20 µl blank urine and 940 µl of 0.2 M

phosphoric acid (Fisherbrand). Final concentration of PABA calibrators were 0.2 µg/ml, 2.5 µg/ml, and 5.0 µg/ml. The calibrators of the metabolites were prepared in the same manner except NaOH was not added during the preparation. Final metabolite calibrators considering a 50 fold dilution of blank urine had final concentrations of 0.2 µg/ml, 2.5 µg/ml, and 5.0 µg/ml.

Experiment Optimization

The PABA quantification was performed using the HPLC method as described by Jakobsen *et al*. Several modifications were made. In that paper, 1.0 ml of urine was subjected to alkaline hydrolysis at 121°C for 90 minutes [8]. The urine volume used for the hydrolysis reaction in this study was decreased to 20 µl in order to achieve better throughput and lower cost to accommodate for the larger sample size. In addition, because many experimental details were omitted in the Jakobsen paper, including the composition of calibrators and the heating apparatus used for the hydrolysis reaction [8], the experimental conditions for PABA analysis by HPLC were examined and optimized. Matrix effects were studied by comparing different matrices with the urine spiked calibrators to determine the appropriate calibrator composition. The effectiveness of NaOH and HCl used for hydrolysis reaction was examined in the hydrolysis reaction reagent section. Three different devices used for the hydrolysis were examined, microwave, boiling water bath, and autoclave. The duration of the hydrolysis reaction was also evaluated for reactions heated by different heating devices.

Matrix Effects

Calibrators consisting of different matrices were examined. Three levels of test solution containing 20 µl of 10 µg/ml, 125 µg/ml, and 250 µg/ml PABA solution were made up to 1.0 ml with the following solvent matrix: de-ionized water; 0.2M phosphoric acid; 0.2 M phosphoric acid with 20 µl of blank urine; 0.2 M phosphoric acid with 20 µl of blank urine, and 20µl of 8 M NaOH; and 0.2 M phosphoric acid adding 20 µl urine heated with 20 µl of 8 M NaOH. The final PABA concentrations of the calibrators were 0.2 µg/ml, 2.5 µg/ml, and 5.0 µg/ml. The area counts for these calibrators were plotted and compared with the sample mimic (calibrator consisting of phosphoric acid adding 20 µl urine heated with NaOH).

Hydrolysis Reaction Reagents

Urine samples of three patients were used to determine the effectiveness of NaOH and HCl for the hydrolysis of PABA metabolites. In a screw-capped vial, 20 µl of urine were combined with 20 µl of 8 M NaOH or 8 M of HCl (BDH). The vials were heated for 90 minutes in a boiling water bath and after they had been cooled to room temperature, 960 µl of 0.2 M phosphoric acid were added. The chromatograms for each HCl and NaOH injection were compared.

Hydrolysis Reaction: Microwave

The effectiveness of the microwave heated hydrolysis reaction of metabolites, PAAB, PAH and PAHA was examined using a household microwave (Danby 0.7 cu. ft.

microwave oven, 700 Watts). The associated degradation of PABA was also evaluated. The individual hydrolysis reaction mixture consisted of 20 µl of 8 M NaOH and 20 µl of 50 µg/ml PABA, PAAB, PAH or PAHA in a 2.0 ml screw-capped vial. Vials of each metabolite were subjected to high-power heating in the microwave for 0.5, 1.0, 1.5, and 2 minutes. The vials were cooled to room temperature and 960 µl of 0.2 M phosphoric acid were added. The final mixture was analyzed using HPLC.

Inter-run precision of the microwave heating method was investigated by microwaving five vials with identical composition separately for one minute each: 20 µl of 8 M NaOH and 20 µl of 150 µg/ml PAH. After the vials were removed from the microwave, 960 µl of 0.2 M phosphoric acid were added to the mixture after they had been cooled to room temperature. The percent coefficient of variation (CV) was computed from the PABA concentration obtained.

Hydrolysis Reaction: Water Bath

The time profile of the hydrolysis reaction was studied, and the hydrolysis reaction of metabolite-spiked blank urine containing either PAAB, PAH or PAHA was carried out in a boiling water bath for 8 hours. The degradation of PABA-spiked blank urine was also examined in the boiling water bath for 8 hours. The individual hydrolysis reaction mixture consisted of 20 µl of 8 M NaOH and 20 µl of 100 µg/ml PABA, PAAB, PAH or PAHA in a 2.0 ml screw-capped vial. Vials of each metabolite were incubated in the boiling water bath for 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, and 480

minutes. After the vials had been removed from the water bath, the vials were cooled to room temperature, and 960 µl of 0.2 M phosphoric acid were added. The final mixture was analyzed using HPLC. Three randomly selected participants' urine samples were also subjected to prolonged heating in a boiling water bath in a similar manner by mixing 20 µl of urine with 20 µl of 8 M NaOH. Hydrolysis time profiles for each metabolite and for participants' urine samples were plotted using the amount of PABA recovered as the independent variable.

Two quantification methods were employed to analyze the amount of PABA recovered after the hydrolysis reaction using the boiling water bath, and the results were compared for forty randomly selected participants' urine samples. The hydrolysis reaction involved mixing 20 µl of urine with 20 µl of 8 M NaOH in a screw-capped vial and heated in a boiling water bath for 30 minutes. After the mixture was cooled to room temperature, 960 µl of 0.2 M phosphoric acid were added. After HPLC analysis had been done, the percent PABA recovery was calculated by quantifying the PABA peak alone or by quantifying the combination of PABA and PAH peaks. The comparison was done using a paired-sample t test and a 2x2 contingency table that used the completion criteria of PABA recovery between 85-110%.

Hydrolysis Reaction: Autoclave

The hydrolysis reaction of PAHA was performed by preparing reaction mixtures in triplicate consisting of 20 µl of 8 M NaOH and 20 µl of 100 µg/ml PAHA in a 2.0 ml

screw-capped vial. It was then autoclaved (Steris Amsco Lab 250 laboratory steam sterilizer) for 90 minutes at 121℃. After cooling to room temperature, 960 µl of 0.2 M phosphoric acid were added. The mixture was vortexed for 1 minute and centrifuged for 3 minutes at 2,500 rpm. A 120 minutes autoclaving cycle was also performed in the same manner. The mixture was analyzed using HPLC.

The PABA calibrators were prepared by mixing 20 µl of 8 M NaOH, 20 µl of blank urine and 20 µl of diluted PABA solution. The mixture was autoclaved for 120 minutes at 121℃ and cooled to room temperature. After the addition of 960 µl of 0.2 M phosphoric acid, it was then vortexed for 1 minute and centrifuged for 3 minutes at 2,500 rpm.

For the rest of this study, urine samples of the participants were hydrolyzed by mixing 20 µl of urine with 20 µl of 8 M NaOH in a screw-capped vial and incubated for 120 minutes at 121℃ in the autoclave for the hydrolysis. After the mixture was cooled to room temperature, 960 µl of 0.2 M phosphoric acid was added. It was then vortexed for 1 minute and centrifuged for 3 minutes at 2,500 rpm. The mixture was analyzed with HPLC.

Comparison: Water bath and Autoclave Method

Forty randomly selected participants' urine samples were hydrolyzed using a boiling water bath and an autoclave with the conditions described above, and the results were compared using the paired-sample t test.

To further test the performance of the two methods, PABA and its metabolites were added to the blank urine, and then were subjected to heating using either autoclave or boiling water bath. In a screw-capped vial, 20 µl of 8 M NaOH and 20 µl of blank urine were mixed with 20 µl of PABA, PAAB, PAH or PAHA solution. Two concentration levels were used, 10 and 250 µg/ml for PABA and 25 and 250 µg/ml for the metabolites. Ten replicates of each concentration were prepared and subjected to autoclaving for 120 minutes at 121℃ or heating in the boiling water bath for 30 minutes. The samples were then cooled to room temperature and 940 µl of 0.2 M phosphoric acid were added. The concentration of PABA was determined for the autoclaved samples while the concentrations of both PAH and PABA were quantified for the water bath samples. The imprecision of the two heating methods was examined.

Three participants' urine samples were also hydrolyzed and analyzed in the same manner, where 20 µl of urine were mixed with 20 µl of 8M NaOH, then heated using autoclave or water bath. The final PABA concentrations were compared using a t test, and the imprecision was examined.

Hydrolysis Reaction: Participants' Urine Samples

For all the participants' 24-hour urine collection, the following experimental procedures were used: In a screw-capped vial, 20 µl of urine were mixed with 20 µl of 8M NaOH and centrifuged for 2 minutes. The vials were autoclaved for 120 minutes at 121℃. The samples were then cooled to room temperature and 960 µl of 0.2 M

phosphoric acid were added. The samples were vortexed for 1 minute and centrifuged for 10 minutes. The samples were then analyzed using HPLC.

Method Validation

Method validation was conducted according to recommendations of the Clinical and Laboratory Standards Institute (CLSI) [64, 65]. The linearity of PABA, PAH and PAHA was evaluated by analyzing three identical samples at six concentrations ranging between 0.2 μg/ml – 10 μg/ml [64]. Each sample contained 20 μl of diluted PABA, PAH or PAHA solution and 980 μl of 0.2 M phosphoric acid. Linear and polynomial regression analyses were performed on the calibration curve.

Imprecision of the instrument was assessed by performing twenty repeated measurements in the same vial containing either 0.2 μg/ml or 10 μg/ml of PABA solution prepared with 0.2 M phosphoric acid. Overall imprecision was determined by assaying three PABA concentration levels (0.2 μg/ml – 10 μg/ml) in triplicates for five days. The inter-day and intra-day mean, standard deviation and coefficients of variation were calculated [65]. Accuracy was evaluated by analyzing two sets of urine PABA quality control (QC) samples at two concentration levels (2.0 μg/ml and 4.0 μg/ml) two times for five days. One set of QC samples was autoclaved for 120 minutes at 121℃. A different batch of PABA (Sigma) was used for preparing QC samples.

Limit of detection (LOD) of PABA was determined to be the concentration at which the response of the analyte was three times that of the noise. The limit of

quantification (LOQ) was determined in a similar manner where response of the analyte was ten times that of the noise.

The overall stability of PABA in urine in the autoclave method was examined by selecting seven participants' urine samples that were received simultaneously, and their PABA concentrations were analyzed at ten time points over a two months period.

C. Sodium and PABA

Urinary sodium was determined by indirect potentiometry using the Beckman Coulter Synchron Clinical System. Analysis of sodium was conducted in the Clinical Trials Clinical Research and Proteomics Laboratory at the Hamilton General Hospital. For PURE24USE, PABA analysis described above was used to evaluate the completeness of urine collection. Samples deemed complete by PABA (85-110% recovery) were not adjusted for sodium levels. For samples containing 70-85% of recovered PABA, the sodium level was adjusted by using the following equation: adjusted 24-hour sodium excretion = measured sodium excretion X (93 / % PABA recovery) [66]. Samples containing less than 70% of recovered PABA were considered incomplete and were excluded from the PURE24USE study.

D. Statistical Methods

All analyses were conducted with an alpha level of $p < 0.05$ using SPSS statistical software (version 20.0). Descriptive statistics were used for demographic values. The overall agreement, sensitivity, specificity, positive predictive value (PPV), and negative

predictive value (NPV) of 24-hour urine collection completeness judged by PABA recovery (85-110%), and participant self-assessments were calculated using direct computation from two by two tables. The relationship between the two criteria was also assessed using Cramer's V coefficient and kappa. The two questions were also used by applying the AND rule and the OR rule. For the AND rule, the participants' samples were considered complete when the participants' responses to both questions were complete. If one or both of the responses to the questions were incomplete, then the participants' samples were considered incomplete. For the OR rule, the participants' samples were considered as complete if their response to one or both of the questions was complete. If the responses to both questions were incomplete, then the participants' samples were considered incomplete.

Subgroup analysis on age, gender and level of education was performed to determine their effect on the PABA recovery. For gender an independent sample t test was used. One-way ANOVA was done for three age groups and four levels of education.

The average sodium excretion was compared based on age and sex using one-way ANOVA after taking BMI into consideration. The sodium level was first log transformed to achieve a normal distribution required for the statistical analysis.

IV. RESULTS

A. Participants

Of the total 1,700 participants from PURE24USE, 681 participants were included in this study because they were aged 37-66 years, and ingested the last PABA pill at the prescribed time (Figure 7). The 339 men and 342 women included in this study had an average age of 56 ± 7 years (Table 1). 612 participants had PABA recoveries between 70 and 110% and an average adjusted urinary sodium of 3,673 ± 1,637 mg/day (mean ± SD). Average PABA recovery was 88.4 ± 15.8% (n=681), with average duration of collection time 24.6 ± 1.3 hours. Of the 681 participants who were eventually included in this study, 438 (64.3%) had complete 24-hour urine collections, according to their urinary PABA recovery (85-110%). The majority of the study population had secondary education (37%) or higher (62%).

IV. RESULTS

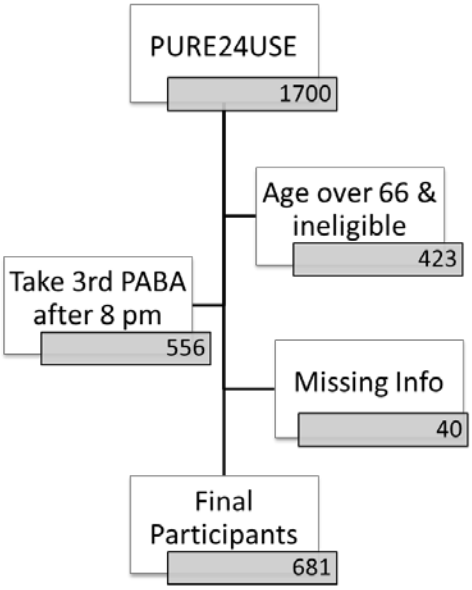

Figure 7: Flow diagram for this study

Table 1: Study participant characteristics. Values are mean ± SD.

Subjects (number)	681
Age (years)	56 ± 7
BMI (kg/m^2)	29 ± 13
Level of Education (number)	
< High School	7
High School	191
Post Secondary	483
Urine volume (mL)	2234 ± 920
Collection time (hours)	24.6 ± 1.3
PABA recovery (%)	88.4 ± 15.8
Sodium (mg/day)	3673 ± 1637

B. PABA Analysis Optimization

Validation of Urine Volume Measurement

The ruler method was validated and found to be statistically different from measurements with a graduated cylinder ($t(41) = 3.96$, $p = 0.0003$). A constant bias of 13.5 ml was found (Figure 8 8), which was equal to 2.1% and 0.2% of the lowest and highest urine volumes respectively. The two measuring methods were found to be highly correlated with a slope of 1.002 (95% CI: 0.994-1.009), $r^2 = 0.9995$ ($p < 0.0001$) (Figure 9). The inter-person (n=4) analysis also showed that the two methods were highly correlated with a slope of 1.002 (95% CI: 0.980-1.025), $r^2 = 0.9994$ ($p < 0.0001$) (Figure 10). The average inter-person bias was 35.4 ± 13.0 ml, with an $r^2 = 0.9998$ ($p < 0.05$). Because the bias observed was not clinically significant, and the volume measured using the ruler agreed very well with that of a graduated cylinder, the ruler method was used for the 24-hour urine volume measurement in this study.

IV. RESULTS

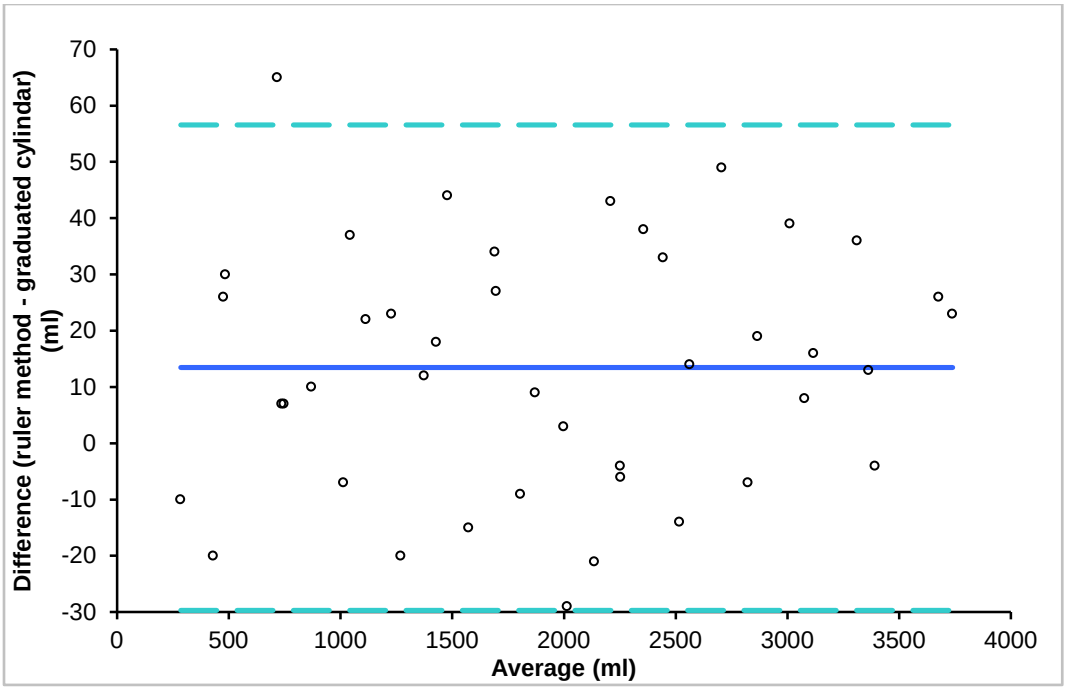

Figure 8: Difference plot for water volume measured by graduated cylinder and ruler method. Bias is shown as the solid line and was determined to be 13.5 ml. Upper and lower 95% limits of agreement are shown as dashed line. (n = 42)

IV. RESULTS

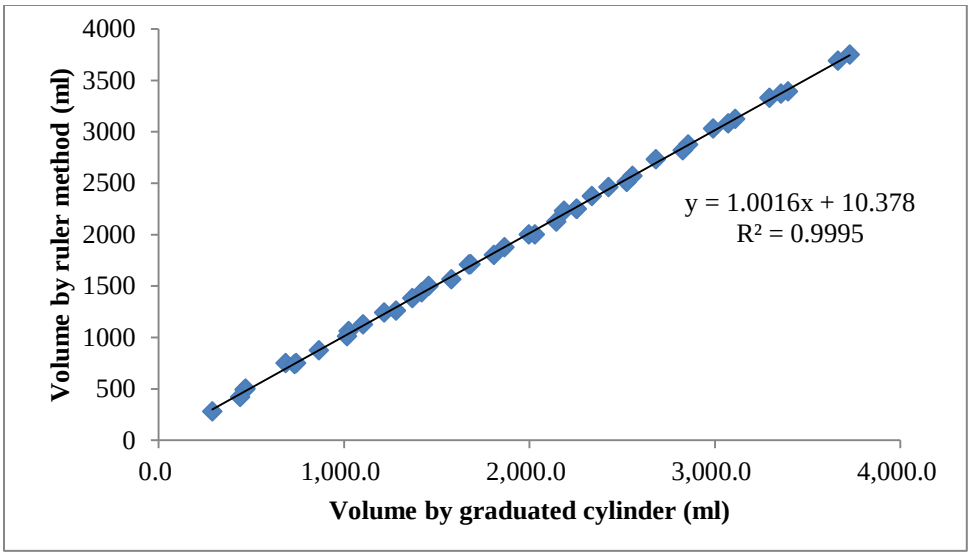

Figure 9: Scatter plot of water volume measured by graduated cylinder and ruler method. The slope was 1.002 (95% CI: 0.994-1.009), with an intercept of 10.378 ml and r^2 of 0.9995 ($p < 0.0001$).

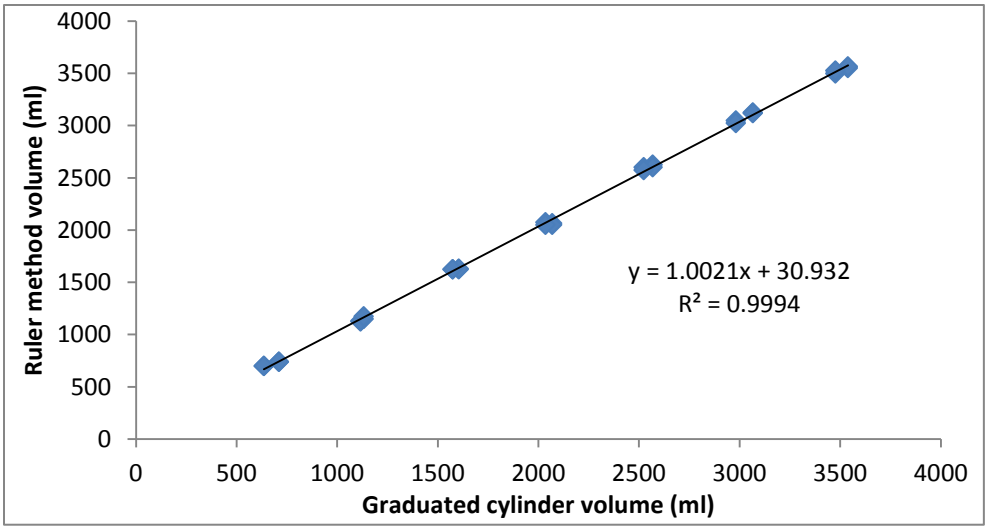

Figure 10: Scatter plot of water volume measured by graduated cylinder and ruler method by four laboratory technicians. The slope was 1.002 (95% CI: 0.980-1.025), with an intercept of 30.932 ml and r^2 of 0.9994 ($p < 0.0001$).

Matrix Effect

Five different calibrator compositions were evaluated and shown in Figure 11, which plotted the calibration curve for each solvent composition using the mean area count from triplicate injections. The addition of blank urine in the calibrator solutions greatly changed the response of the analyte at higher concentration. The difference was found to be statistically significant ($t(2) = 65.4$, $p = 0.0002$). The use of calibrators that did not contain urine resulted in underestimation of PABA concentration. Three sets of calibrators that contained urine behaved similarly. However, the calibrators had to be heated to closely mimic the real urine sample. Because the calibrator solvent consisting of 0.2 M phosphoric acid, 20 µl blank urine and 20 µl 8 M NaOH best mimicked the urine sample, it was used for the rest of the experiment except for the hydrolysis experiment using the autoclave.

IV. RESULTS

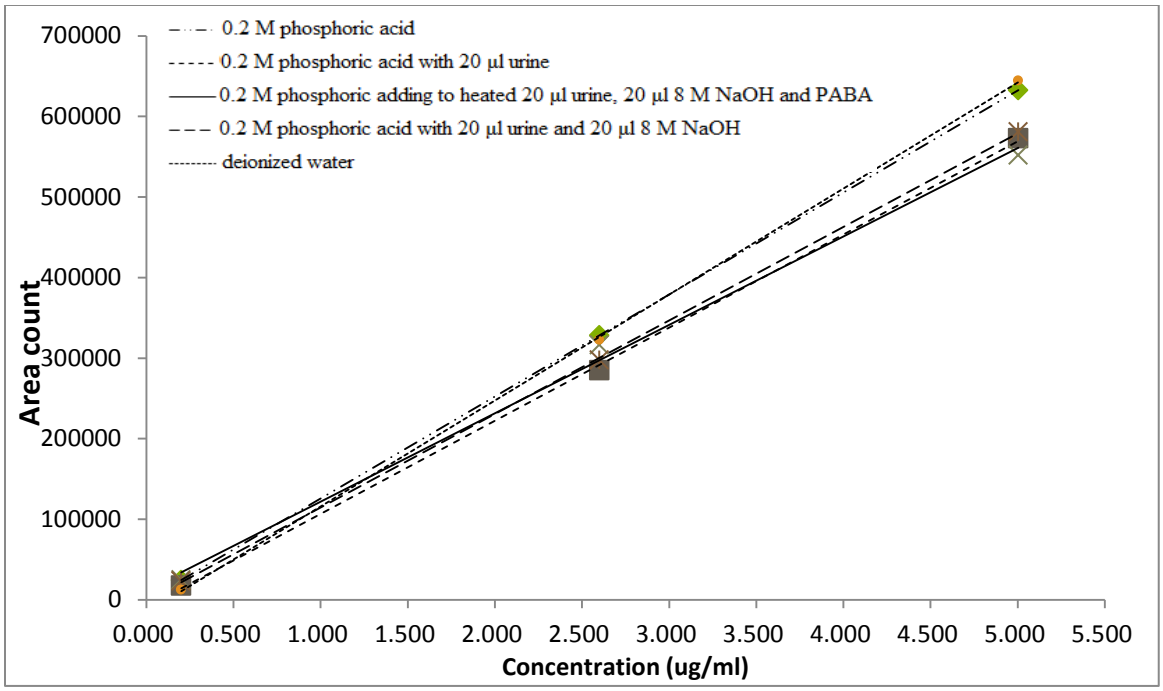

Figure 11: Calibration Curve for PABA calibrators of different composition, measured in triplicate.

Hydrolysis Reaction Reagent

The usefulness of HCl in the hydrolysis reaction was assessed by comparing three hydrolyzed urine samples, and is shown in Figure 12. Contrary to the sharp and symmetrical PABA peaks found in the NaOH hydrolyzed samples, broader and unsymmetrical PABA peaks were observed. The calculated concentration for one of the samples hydrolyzed using HCl was 1.73 fold higher than that of NaOH. The other two samples hydrolyzed using HCl had slightly lower concentration than that of NaOH. For

the rest of this study, NaOH was used for the hydrolysis reaction because of better peak shapes.

IV. RESULTS

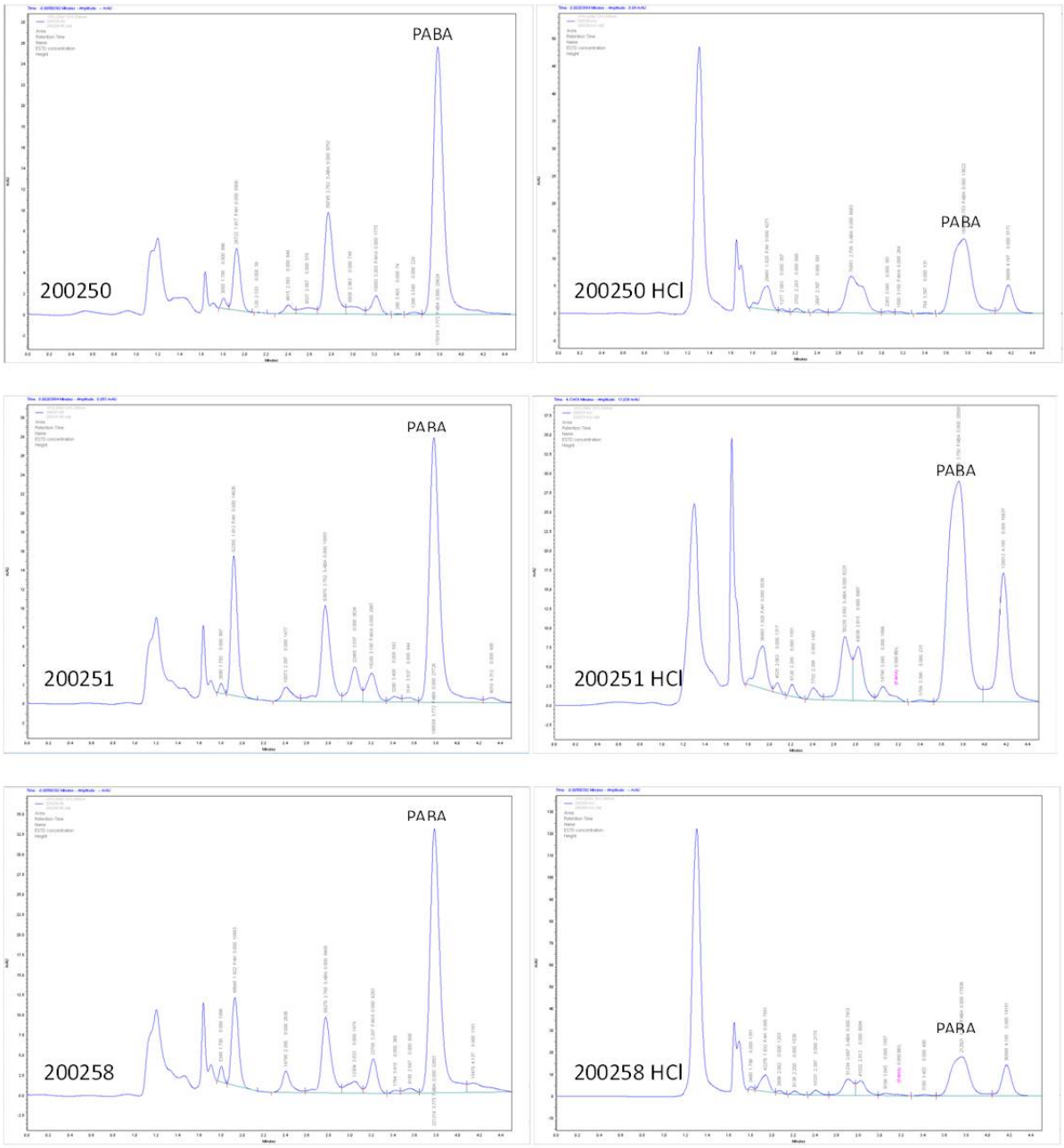

Figure 12: Chromatograms of patient urine sample hydrolyzed using either NaOH (left) or HCl (right).

IV. RESULTS

Hydrolysis Reaction: Microwave

Figure 13 shows the hydrolysis reaction progress of PABA metabolites over time in the microwave. The conversion of the metabolites to PABA reached a maximum at 1.2 minutes with the exception of PAHA. However, because the yield of the reaction over time was more variable than expected, the intra-run precision of the microwave was tested. The CV was 47% for five identical samples microwaved separately. Therefore, although a microwave can be a fast and convenient method for the hydrolysis reaction, the low precision made it an undesirable method.

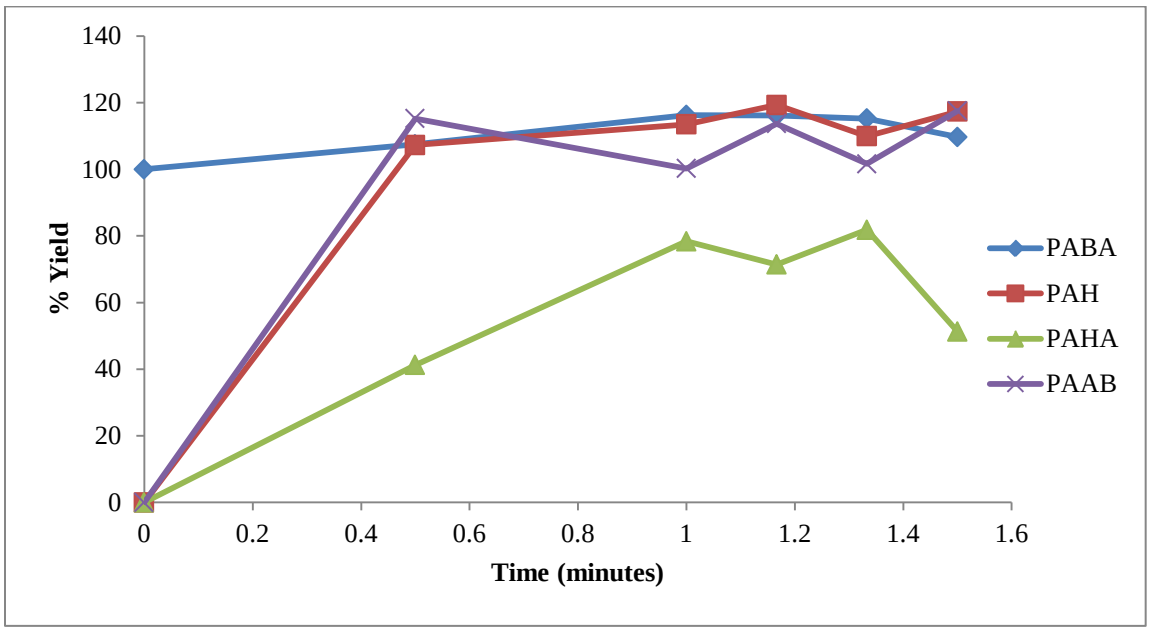

Figure 13: Hydrolysis reaction progress for PABA, PAAB, PAH and PAHA.

Hydrolysis Reaction: Water Bath

The effectiveness of the hydrolysis reaction for PABA metabolites in a boiling water bath was investigated (Figure 14). PAAB easily hydrolyzed to PABA while PAH and PAHA were found to hydrolyze at a very slow pace. In addition, PAH was observed in the reaction vessel of PAHA at the end of the hydrolysis reaction. Over the eight hour heating period, there was up to 10% degradation of PABA. A similar experiment on three urine samples showed maximum PABA recovery of 90% occurring within the first 90 minutes (Figure 15). Up to 10% degradation was observed when the samples underwent prolonged heating. Therefore, a reaction time of 30 minutes was chosen as an optimum.

The hydrolysis reaction never reached completion for the conversion of all PABA metabolites to PABA. The deacetylation of PAHA and PAAB occurred rapidly and after 30 minutes, the reaction vials were left with PAH and PABA. Therefore, for the rest of the hydrolysis reaction using the water bath in this study, the quantification included both the PABA and the PAH peak on the chromatogram.

IV. RESULTS

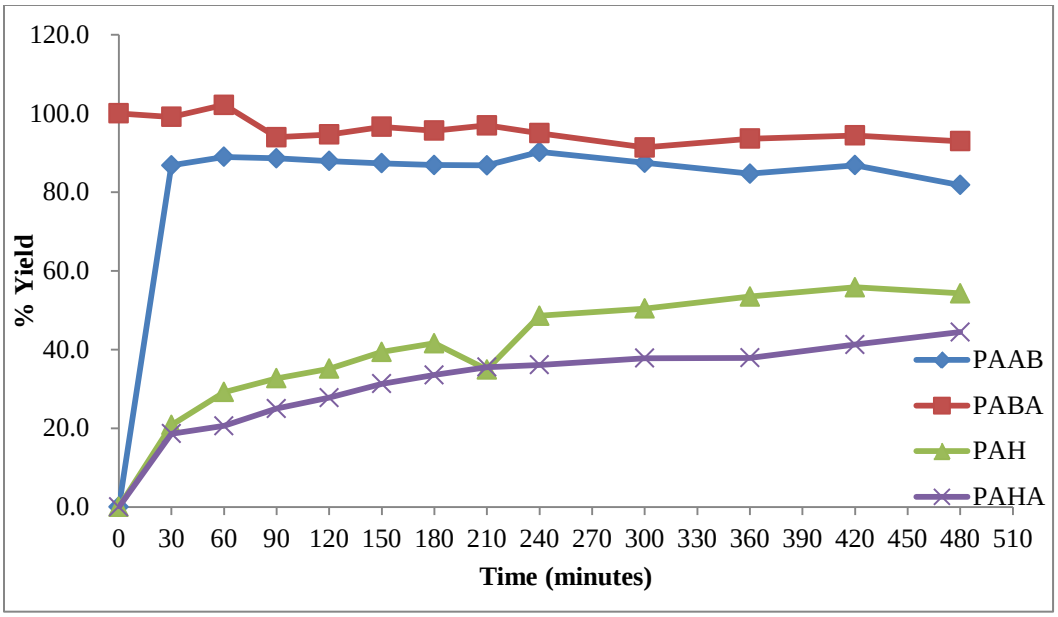

Figure 14: Hydrolysis yield for PAAB, PAH and PAHA during an eight hour heating in boiling water bath. The degradation profile of PABA is also shown. The percent PABA recovery was determined every half hour for the first four hour and every hour after that.

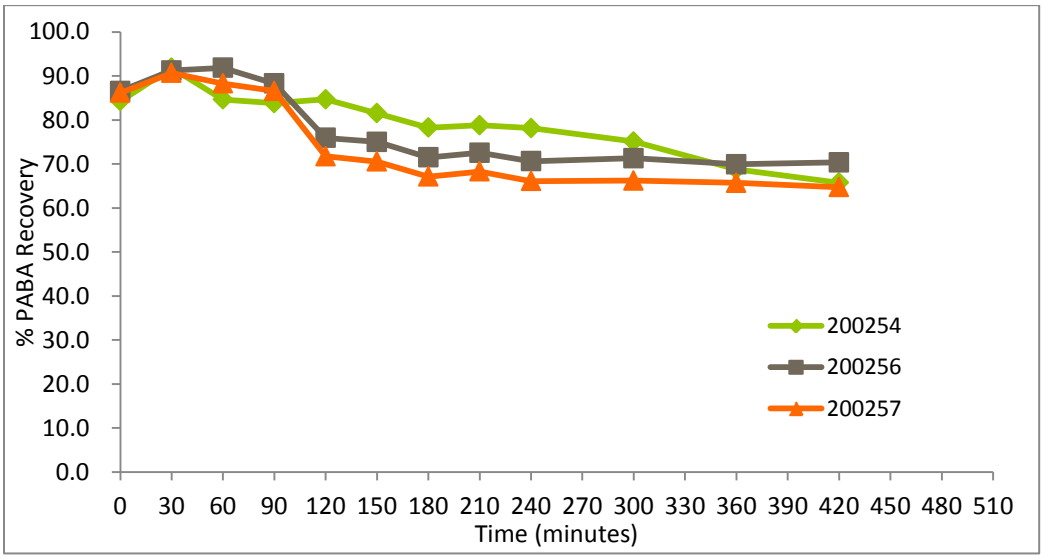

Figure 15: Percent PABA recovery for participants' urine samples that underwent eight hours of heating in a boiling water bath.

IV. RESULTS

Forty urine samples were analyzed by applying the above described experimental settings, and the results of the two quantification methods (PABA vs PABA + PAH quantitation) were found to be significantly different ($t(39) = 10.49$, $p < 0.0001$). The PABA recovery increased by an average of 21.5 ± 2.8% SE when the metabolite PAH was included into the quantification of the PABA recovery. In addition, when the completeness of the urine collections was determined based on PABA recovery, 60% of the samples were incorrectly identified as incomplete (Table 2).

Table 2: Classification of participants' urine collection as complete or incomplete based on quantification of PABA and of PABA plus PAH ($p = 0.708$)

			Quantified with PABA and Metabolites		
			Complete Collection	Incomplete Collection	Total
Quantified with PABA	Complete Collection	Count	1	1	2
		% within Quantified with PABA	50.0%	50.0%	100.0%
	Incomplete Collection	Count	24	14	38
		% within Quantified with PABA	63.2%	36.8%	100.0%
Total		Count	25	15	40
		% within Quantified with PABA	62.5%	37.5%	100.0%

Hydrolysis Reaction: Autoclave

PAHA was used as an indicator for the completeness of the hydrolysis reaction since it had the lowest hydrolysis rate. When a 90 minutes autoclave cycle was used for the hydrolysis of PAHA, the yield of the reaction was found to be 51%. In addition, when the resulting chromatogram was examined, a PAH peak was seen. Therefore the reaction

time was increased to 120 minutes. After heating for 120 minutes, the PAHA hydrolysis yield was 99.9% and considered complete. The stability of PABA was not affected by these reaction conditions.

PABA calibrators were also subjected to a 120 minutes autoclave cycle to determine the effect of high pressure and temperature on the calibration in comparison to un-autoclaved calibrators. Quantitation of study samples was more precise with autoclaved vs un-autoclaved calibrators (Tables 7, 8). Therefore, PABA calibrators were autoclaved for the rest of the experiment.

Water bath and Autoclave Comparison

Forty patients' urine samples were subjected to hydrolysis heating using the autoclave and the boiling water bath. The calibrators were heated by either autoclave or the boiling water bath. No statistically significant difference ($t(39) = 0.567$, $p = 0.574$) was observed for the results obtained using two methods.

Two concentration levels and ten replicates of PABA or metabolites spiked into blank urine were used to determine the accuracy and imprecision of the two hydrolysis methods (Table 3). Both methods showed similar acceptable yields and CVs.

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Table 3: Hydrolysis yield for PABA and metabolites, heated with autoclave or water bath. Two concentration levels were used with ten replicates for each level.

0.5 µg/ml	PABA		PAAB		PAH		PAHA	
Method	Water bath	autoclave	Water bath	autoclave	Water bath	autoclave	Water bath	autoclave
% Yield	84	95	85	90	91	96	93	95
% CV	3.8	7.5	2.7	5.7	6.8	4.5	7.3	4.9
5.0 µg/ml	PABA		PAAB		PAH		PAHA	
Method	Water bath	autoclave	Water bath	autoclave	Water bath	autoclave	Water bath	autoclave
% Yield	104	98	89	95	110	98	108	87
% CV	2.9	1.8	2.7	10.3	4.2	3.8	3.1	2.3

Three participants' urine samples were also hydrolyzed in autoclave and water bath in ten replicates, with similar precision (Table 4).

Table 4: Patient urine samples hydrolyzed in water bath or autoclave. The results of ten replicates experiments were shown as average, standard deviation and percent CV. The results calculated using two methods were not statistically different.

ID	Method	Mean ± SD (µg/ml)	% CV	t test	p value
200229	water bath	0.64 ± 0.01	2.07	0.52	0.615
	autoclave	0.65 ± 0.03	4.59		
200230	water bath	1.10 ± 0.03	3.15	1.20	0.260
	autoclave	1.08 ± 0.04	3.50		
200231	water bath	2.62 ± 0.06	2.31	0.94	0.369
	autoclave	2.65 ± 0.07	2.81		

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Method Validation

The linearity of PABA and PAH quantitation by HPLC (Figure 16) was evaluated using linear and polynomial regression analysis. PABA was found to be linear over the range of 0.2 µg/ml – 10 µg/ml with r^2 = 0.9996 (Figure 17). PAH was found to be linear from 0.13 µg/ml to 2.72 µg/ml with r^2 = 0.9959 (Figure 18). None of the non-linear coefficients were found to be statistically significant according to the polynomial regression method.

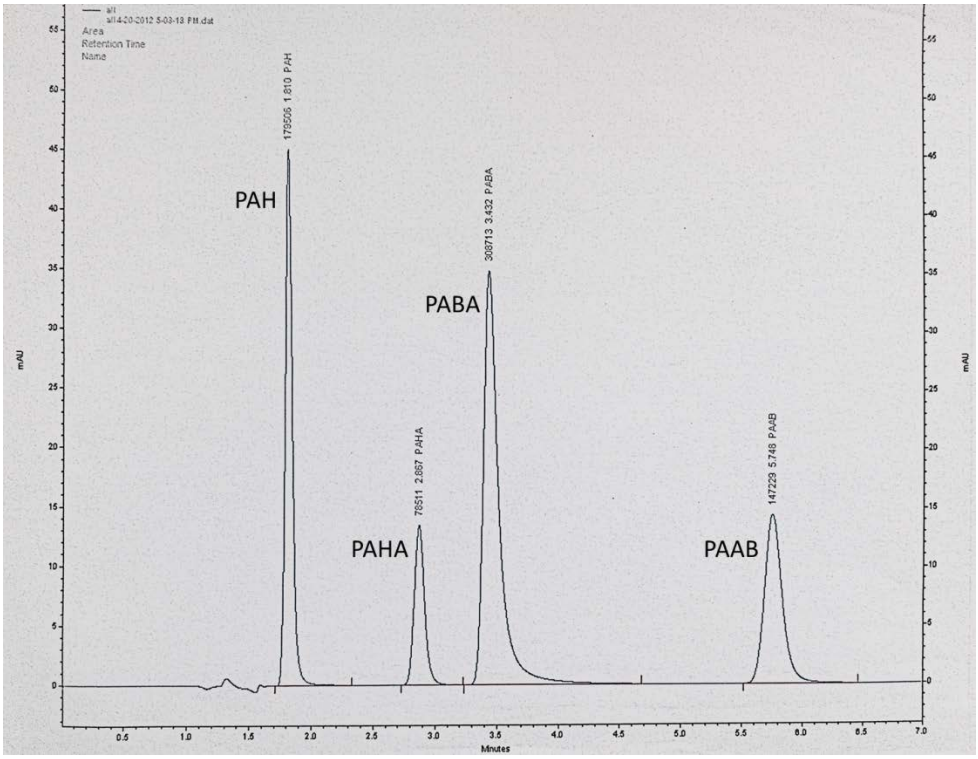

Figure 16: Chromatogram of PABA and its metabolites

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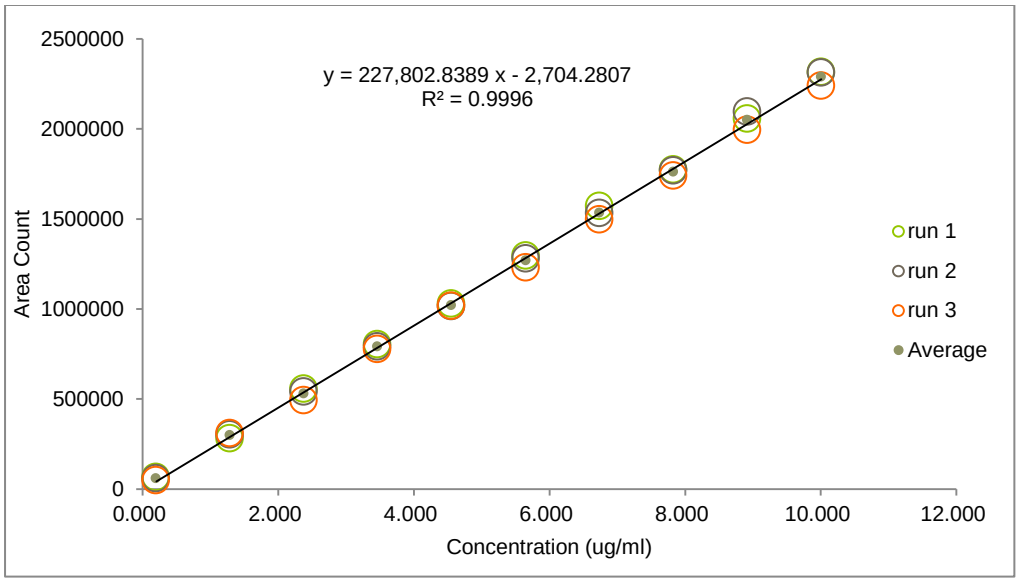

Figure 17: Calibration curve for PABA. The circles represent three replicate solutions at each concentration level. The linear regression line was calculated using average of the three runs.

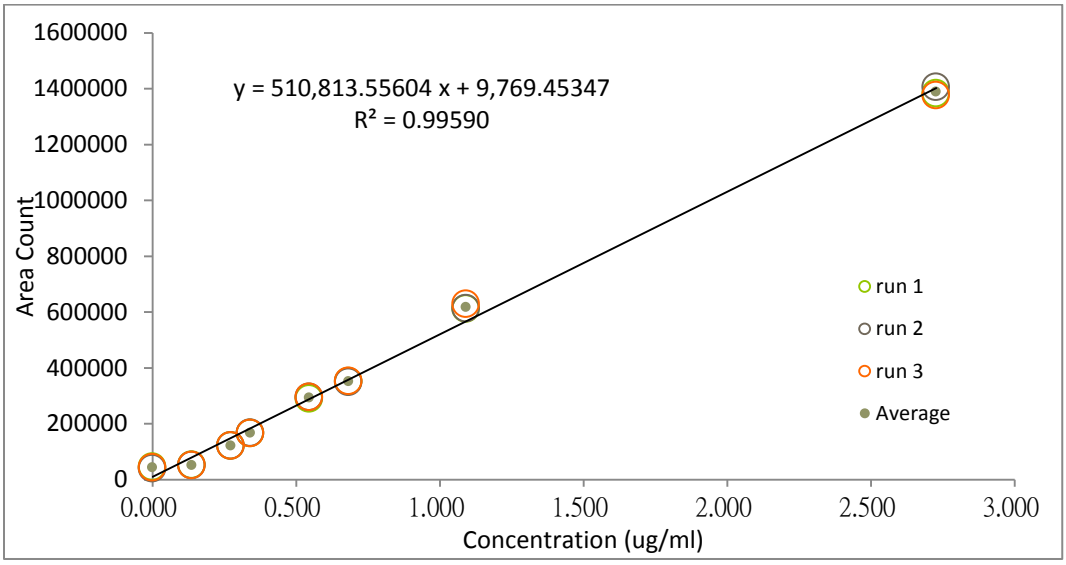

Figure 18: Calibration curve for PAH. The circles represent three replicate solutions at each concentration level. The linear regression line was calculated using average of the three runs.

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The instrument precision was evaluated using 20 injections of a single PABA solution at two concentration levels. The percent CV was 8.7% at 0.2 μg/ml and 1.9% at 10 μg/ml. Inter-day and intra-day precision and inter-day accuracy is summarized in Table 5. The precision study was carried out for five days at three concentration levels (0.5 μg/ml, 5.0 μg/ml, and 10 μg/ml).

Table 5: Imprecision associated with PABA quantification using HPLC. Inter-day and Intra-day precision and inter-day accuracy for PABA at three concentration levels, analyzed in triplicate. Values are mean ± SD.

Concentration (μg/ml)	0.5004	5.004	10.008
Day	mean	mean	mean
1	0.600 ± 0.009	5.270 ± 0.10	10.28 ± 0.15
2	0.422 ± 0.020	5.17 ± 0.03	10.37 ± 0.11
3	0.515 ± 0.020	4.96 ± 0.03	9.84 ± 0.10
4	0.446 ± 0.050	4.95 ± 0.10	9.71 ± 0.17
5	0.429 ± 0.0005	5.29 ± 0.06	10.27 ± 0.35
Mean (μg/ml)	0.485 ± 0.075	5.13 ± 0.16	10.09 ± 0.30
Accuracy (%)	96.4	100.2	100.8
Inter-run % CV	16.3	3.4	3.4

The precision study was also done on autoclaved QC samples at two concentration levels for five days, as shown in Table 6. The LOD and LOQ for PABA were 0.025 μg/ml and 0.087 μg/ml respectively. The LOD and LOQ for PAH were 0.070 μg/ml and 0.120 μg/ml respectively. PABA in urine using the autoclave method used for the hydrolysis reaction was found to be stable, with an imprecision that ranged between 2-4% coefficients of variation (Table 7) when autoclaved calibrators were used for

concentration calculation. When unheated calibrators were used, the imprecision ranged between 8-12% coefficients of variation (Table 8).

Table 6: Imprecision associated with unheated and autoclaved PABA. Inter-day and Intra-day precision and inter-day accuracy for PABA at two concentration levels that were analyzed two times for five days. Values are mean ± SD.

Concentration (μg/ml)	2.09		4.18	
Method / Day	Original	Autoclaved	Original	Autoclaved
1	2.25 ± 0.02	2.14 ± 0.01	4.51 ± 0.07	4.09 ± 0.07
2	2.05 ± 0.08	2.04 ± 0.05	4.05 ± 0.001	3.98 ± 0.17
3	2.02 ± 0.04	2.08 ± 0.09	4.06 ± 0.13	3.96 ± 0.12
4	2.19 ± 0.15	1.89 ± 0.15	4.22 ± 0.01	3.93 ± 0.14
5	2.14 ± 0.05	2.06 ± 0.05	4.00 ± 0.06	4.34 ± 0.05
Mean (μg/ml)	2.13 ± 0.09	2.04 ± 0.10	4.17 ± 0.21	3.99 ± 0.07
Accuracy (%)	102.0	97.6	99.8	95.6
Inter-run % CV	4.4	5.1	5.0	1.7

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Table 7: Imprecision associated with autoclaved samples. Seven autoclaved participants' urine samples were analyzed using autoclaved calibrator at ten randomly selected days.

	PABA Concentration (μg/ml)												
ID	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Mean	SD	% CV
300079	2.55	2.28	2.32	2.29	2.32	2.39	2.32	2.34	2.28	2.29	2.34	0.08	3.53
300081	6.02	5.99	6.22	6.17	6.31	6.04	5.57	6.23	6.13	6.32	6.10	0.22	3.60
300084	1.54	1.54	1.51	1.52	1.56	1.59	1.66	1.57	1.58	1.60	1.57	0.04	2.83
300085	3.05	3.10	3.18	3.00	3.23	3.24	3.18	3.20	2.98	3.21	3.14	0.10	3.15
300086	1.03	0.95	0.94	0.94	0.94	1.00	1.02	0.96	0.94	0.94	0.97	0.04	3.87
300087	2.96	2.91	2.99	2.90	3.12	3.09	2.98	3.00	3.00	3.10	3.00	0.08	2.51
300088	1.30	1.27	1.30	1.27	1.24	1.30	1.28	1.28	1.22	1.26	1.27	0.03	2.13

Table 8: Imprecision associated with calibrators. Seven autoclaved participants' urine samples were analyzed using unheated calibrator at ten randomly selected days.

	PABA Concentration (μg/ml)												
ID	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Mean	SD	% CV
300079	1.83	2.39	1.89	2.64	2.27	2.27	2.30	2.23	2.28	2.40	2.25	0.24	10.52
300081	4.65	6.31	5.00	7.01	6.06	5.66	6.17	5.89	6.14	6.51	5.94	0.69	11.68
300084	1.27	1.58	1.27	1.69	1.55	1.53	1.52	1.57	1.61	1.55	1.52	0.14	8.94
300085	2.45	3.25	2.46	3.51	3.14	3.06	3.15	2.89	3.16	3.21	3.03	0.34	11.21
300086	0.83	1.00	0.81	0.99	0.97	0.98	0.92	0.96	0.98	0.90	0.93	0.07	7.29
300087	2.31	3.07	2.38	3.33	3.03	2.92	2.95	2.92	3.05	3.10	2.91	0.32	10.97
300088	1.07	1.37	1.07	1.38	1.25	1.26	1.24	1.23	1.28	1.25	1.24	0.10	8.25

C. Completeness of 24-Hour Urine Collection

No association was found for the response to the question "During the 24-hour collection period, did you always void into the urine collection container," and the PABA recovery in regard to determining the completeness of 24-hour urine collection (χ^2 = 0.593, p = 0.441) (Table 9), with an overall agreement of 63.4%, while the kappa demonstrated no agreement between the two methods used to evaluate completeness of urinary collection, κ = 0.017 (95% CI: -0.027– 0.065), p = 0.476. The estimated sensitivity for the completeness of 24-hour urine collection assessed by participants using the above question, compared with reference standard was 95.2% (95% CI: 93.9%-96.6%), with an estimated specificity of 6.2% (3.9%-8.7%), a PPV of 64.7% (95% CI: 63.8%-65.6%) and a NPV of 41.7% (26.2%-58.7%). This question had a likelihood ratio of 0.581 (p = 0.446).

Table 9: Completeness of urine collection based on participants' responses. Urinary completeness was based on PABA recovery and the participants' responses to the question, "During the 24-hour collection period, did you always void into the urine collection container." (n = 681).

		Complete 24hour urine collection (PABA 85-110)		
		Yes	NO	Total
During 24 hr collection did you always void into urine collection container?	Complete	417 64.7%	228 35.3%	645 100.0%
	Incomplete	21 58.3%	15 41.7%	36 100.0%
Total		438 64.3%	243 35.7%	681 100.0%

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When Question 2, "Is any urine missing from the collection for any other reason" was used, there was evidence of association to that of the PABA recovery in regard to determining the completeness of 24-hour urine collection ($\chi^2 = 6.026$, p= 0.014) (Table 10). The association was found to be weak (Cramer's V = 0.094, p = 0.014) between the answer and PABA recovery. The observed agreement between the two was 60.8%, while the kappa demonstrated slight agreement between the two, $\kappa = 0.092$ (95% CI: 0.015–0.170), p = 0.038. The estimated sensitivity for the completeness of 24-hour urine collection assessed by participants compared with PABA standard was 76.9% (95% CI: 74.4%-79.6%), with an estimated specificity of 31.7% (95% CI: 27.0%-36.4%), a PPV of 67.0% (95% CI: 64.7%-69.3%) and an NPV of 43.3% (95% CI: 36.9%-49.7%).This question had a likelihood ratio of 5.928 (p = 0.015).

Table 10: Completeness of urine collection based on participants' responses. Urinary completeness was based on PABA recovery and the participants' response to the question "Is any urine missing from the collection for any other reason." (n = 681)

		Complete 24hour urine collection (PABA 85-110)		
		Yes	NO	Total
Is any urine missing from collection	Complete	337	166	503
		67.0%	33.0%	100.0%
	Incomplete	101	77	178
		56.7%	43.3%	100.0%
Total		438	243	681
		64.3%	35.7%	100.0%

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When both questions were used together using the AND rule, there was an evidence of association to that of the PABA recovery in regard to determining the completeness of 24-hour urine collection ($\chi^2 = 6.326$, $p = 0.012$) (Table 11). The association was found to be weak (Cramer's $V = 0.096$, $p = 0.012$) between the answers and PABA recovery. The observed agreement between the two was 60.6%. The estimated sensitivity for the completeness of 24-hour urine collection assessed by participants compared with PABA standard was 76.0%, with an estimated specificity of 33.0%, a PPV of 67.1% and an NPV of 43.2%. The AND rule had a likelihood ratio of 6.227 ($p = 0.013$).

Table 11: Completeness of urine collection based on participants' responses. Urinary completeness was based on PABA recovery and the AND rule (n = 681).

		Complete 24hour urine collection (PABA 85-110)		
		Yes	NO	Total
Questionnaire Questions	Both Complete	333	163	496
		67.1%	32.9%	100.0%
	Incomplete	105	80	185
		56.8%	43.2%	100.0%
Total		438	243	681
		64.3%	35.7%	100.0%

No association was found for the OR rule and the PABA recovery in regard to determining the completeness of 24-hour urine collection ($\chi^2 = 0.528$, $p= 0.513$) (Table 12), with an overall agreement of 63.6%. Using the OR rule, the combined sensitivity

was 95.2%, specificity was 2.0%, PPV 64.6% and NPV 41.4%. The OR rule had a likelihood ratio of 0.420 (p = 0.517).

Table 12: Completeness of urine collection based on participants' responses. Urinary completeness was based on PABA recovery and the participants' responses when OR rule was applied (n = 681).

			Complete 24hour urine collection (PABA 85-110)		
			Yes	NO	Total
ORrule	Complete	Count	421	231	652
		% within ORrule	64.6%	35.4%	100.0%
	Incomplete	Count	17	12	29
		% within ORrule	58.6%	41.4%	100.0%
Total		Count	438	243	681
		% within ORrule	64.3%	35.7%	100.0%

Subgroup analysis was conducted on gender and age. No significant difference was observed in percent PABA recovery between genders ($t(679) = 1.767$, $p = 0.078$), with men having an average of 89.5 ± 14.3% and women 87.3 ± 17.1%. The ratio of true positives and true negatives for Question 1 was roughly the same for both males and females. However, for Question 2, the specificity for males (16.7%) was much lower than that of the females (43.7%), and the sensitivity was much higher for males (87.0%) than for females (65.7%) (Table 13). These were also observed when the AND rule was used.

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Table 13: The contingency table of urinary completeness based on PABA recovery and the participants' response to the question "Is any urine missing from the collection for any other reason," by gender (n = 681).

Gender			Complete 24hour urine collection (PABA 85-110)		Total
			Yes	NO	
Male	Is any urine missing from collection	Complete	201	90	291
			69.1%	30.9%	100.0%
		Incomplete	30	18	48
			62.5%	37.5%	100.0%
	Total		231	108	339
			68.1%	31.9%	100.0%
Female	Is any urine missing from collection	Complete	136	76	212
			64.2%	35.8%	100.0%
		Incomplete	71	59	130
			54.6%	45.4%	100.0%
	Total		207	135	342
			60.5%	39.5%	100.0%

The mean PABA recovery of the completed urine collections (n = 503) determined by Question 2 was 89.4 ± 15.4% with mean sodium excretion of 3,610 ± 1,607 mg/day. The mean PABA recovery of the completed urine collections (n = 438) determined by PABA recovery was 94.0 ± 5.9% with mean sodium excretion of 3,643 ± 1,625 mg/day.

The participants were stratified into three age groups, 37-53 years (n = 239), 54-59 years (n = 224) and 59-66 years (n = 218) (Table 14). The difference in percent PABA recovery between the three age groups was not statistically significant with $F(2, 678) = 1.57$, $p = 0.258$. In addition, percent PABA recovery was not found to be associated with

age before (b = -0.169, r^2 = 0.07, p = 0.068), and after correction for sex and BMI (b = -0.067, r^2 = 0.014, p = 0.079). Sensitivity and specificity for all three strata were similar.

Subgroup analysis was also performed on the participants' education level. The difference in percent PABA recovery between three education groups was not statistically significant with F(3, 678) = 1.982, p = 0.115. Sensitivity and specificity were similar for all education levels.

Table 14: PABA recovery for different age strata

age	N	Mean (%)	SD	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
37-53	239	89.61	15.497	87.63	91.58
54-59	224	88.32	16.063	86.21	90.44
60-66	218	87.17	15.825	85.06	89.29
Total	681	88.41	15.798	87.22	89.59

Sodium Intake

Based on the methods used to determine urinary collection completeness, the participants' sodium excretion were compared (Table 15). By using the PABA recovery 85%-110%, Question 2, "Is any urine missing from the collection for any other reason," and the AND rule, the differences between sodium excretions for complete and incomplete urinary collections were found to be statistically significant.

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Table 15: Participants' sodium excretion (mg/day), by urinary collection completeness.

PABA 70% - 110%							
70% - 110%			other				
N	Mean	SD	N	Mean	SD	t	p value
612	3510	1581	69	3172	1297	1.716	0.087
Complete 24hour urine collection (PABA 85-110)							
85% - 110%			No				
N	Mean	SD	N	Mean	SD	t	p value
438	3643	1625	243	3174	1378	3.806	0.000
During 24 hr collection did you always void into urine collection container?							
Complete			Incomplete				
N	Mean	SD	N	Mean	SD	t	p value
645	3495	1551	36	3141	1648	1.329	0.184
Is any urine missing from collection							
Complete			Incomplete				
N	Mean	SD	N	Mean	SD	t	p value
503	3577	1585	178	3189	1440	2.873	0.004
AND rule							
Complete			Incomplete				
N	Mean	SD	N	Mean	SD	t	p value
496	3580	1594	185	3198	1420	2.864	0.004
OR rule							
Complete			Incomplete				
N	Mean	SD	N	Mean	SD	t	p value
652	3494	1544	29	3076	1800	1.414	0.158

In this study, 612 of 681 (89.9%) participants had PABA recovery between 70-110%, and the sodium content of their urine samples was included in this study. Of these, 438 participants had complete 24-hour urine and a mean sodium excretion of 3,643 ± 1,625 mg/day (Figure 19). The rest of the 174 participants had incomplete 24-hour urine (PABA recovery 70-84%), and they were also included in the analysis for the

PURE24USE study after the amount of sodium excreted was adjusted according to the equation described in the Method section. A paired sample t test showed that the measured (3,175 ± 1,413 mg/day) and adjusted (3,746 ± 1,668 mg/day) sodium excretions were significantly different, $t(173) = 23.377$, $p < 0.001$. The mean sodium excretion was significantly different between the participants with complete and incomplete collection before the sodium excretion was adjusted, $F(1, 610) = 11.116$, $p = 0.001$. The mean adjusted sodium excretion was not significantly different between the participants with complete and incomplete collection, $F(1, 610) = 0.492$, $p = 0.483$. Finally, the overall measured (3,510 ± 1,581 mg/day), and adjusted (3,673 ± 1,637 mg/day) sodium excretions were significantly different with $t(611) = 12.973$, $p < 0.001$.

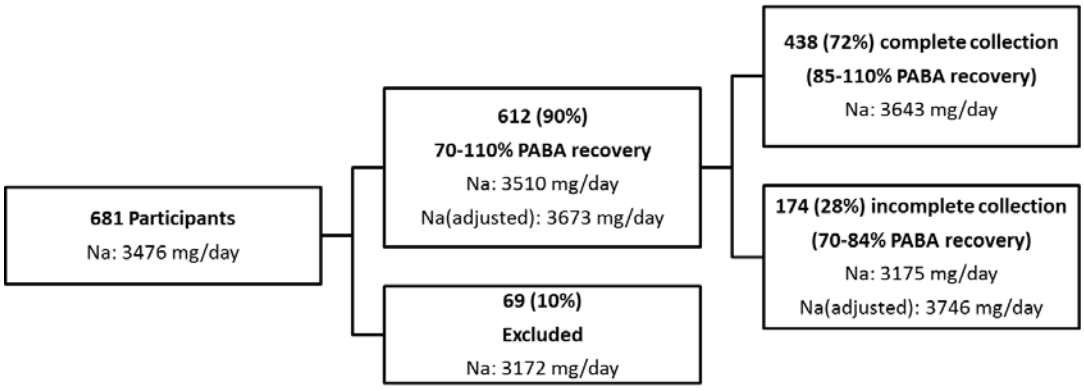

Figure 19: Participants' sodium intake. Mean measured and adjusted sodium excretion for complete and incomplete 24-hour urine collection based on PABA recovery

The subgroup analysis showed that men (4,210 ± 1,742 mg/day) had a significantly higher sodium excretion compared to women (3,139 ± 1,326 mg/day), t(610)

= -8.656, $p < 0.001$. The difference remained statistically significant after correction for age and BMI, $F(1, 609) = 63.323$, $p < 0.001$ (Table 16). The difference in Na excretion between three age groups (age 37-53: 3,909 ± 1,849 mg/day; age 54-59: 3,672 ± 1,628 mg/day; age 60-66: 3,411 ± 1,334 mg/day) showed a downward trend that almost reached statistical significance, ($F(2, 609) = 2.956$, $p = 0.053$). When the BMI and gender were taken into consideration, the difference was found to be statistically significant, $F(2, 607) = 3.755$, $p = 0.024$ (Table 16).

Table 16: Mean value of adjusted sodium excretion, by sex and age group.

Gender	Current Age	Mean	N	SD
Male	37-53	4165	106	1653
	54-59	4240	89	1429
	60-66	3756	101	1303
	Total	4048	296	1484
Female	37-53	3389	109	1501
	54-59	2944	101	1082
	60-66	2991	96	1134
	Total	3117	306	1274

V. DISCUSSION AND CONCLUSIONS

A. Overview of Main Findings

This study optimized an often cited HPLC method for analysis of PABA in urine [8] and adopted the modified method for the validation of completeness of 24-hour urine collection in the PURE24USE study. The effectiveness of participants' self-reported assessment of urinary completeness was compared to the PABA method. The sample preparation was simplified and downscaled fifty times to allow large-scale analysis with supplies typically available in a contemporary clinical laboratory with HPLC equipment. The mobile phase was changed to a citric acid – sodium phosphate dibasic buffer (pH~3.5) to achieve an appropriate buffer capacity and thus stable retention times. The hydrolysis time was lengthened to 120 minutes, and an autoclave was specified as the apparatus used for heating the reaction. It was also shown that instead of an autoclave a boiling water bath can be used with similar results, if only one additional PABA metabolite (PAH) is included in the recovery calculation.

Based on urinary PABA recovery, 612 (89.9%) of the 681 participants' samples were included, and 438 participants (64.3%) had complete 24-hour urine. The overall mean PABA recovery was 88.4 ± 15.8%, with a mean urine collection time of 24.6 ± 1.3 hours. No association was found between PABA recovery and age. Participants' self-reported assessment of urinary completeness was compared with the PABA marker. Sensitivity of self-report to predict PABA was 76.9% (95% CI: 74.4%-79.6%) and

specificity was 31.7% (95% CI: 27.0%-36.4%). This agreement does not support omitting PABA analysis in future studies with the strictest possible criteria of 24-hour urine completeness. Comparisons of questionnaire and PABA to a higher diagnostic accuracy standard – such as directly observed urine collection – were not done in this study. The theoretical possibility remains that self-report was more accurate than PABA. This seems unlikely since PABA is an objective, previously clinically validated marker. An improved method for measuring PABA in large studies was analytically validated in this work.

The mean adjusted sodium intake was 3,673 ± 1,637 mg/day, which was higher than the previous Canadian mean of 3,100 mg/day [67]. Men had significantly higher mean sodium intake than women. No significant difference in sodium intake between different age groups was observed, but there was a tendency of lower excretion at higher age.

B. PABA Analysis

Urine Volume Measurement

For the determination of urine volume in this study, a special ruler and a standard container were used instead of using a graduated cylinder or weighing. There was very good agreement between the results measured using this ruler and that of the graduated cylinder (Figure 9). Although there was a small bias associated with the ruler method (Figure 8), the bias was not found to be clinically significant to affect the results of the

study. Furthermore, the ruler method can be adopted by different laboratory personnel because it is found to have a low inter-person imprecision (Figure 10). Using the ruler method has advantages over the other two measuring methods. Besides being direct and simple, it does not require pouring or additional laboratory equipment. Therefore the ruler method can effectively replace the graduated cylinder or weighing for the accurate measurement of urine volume, and was used for measuring 24-hour urine collection in this study.

PABA Analysis – HPLC settings

PABA has been implemented by many epidemiological and clinical studies to validate urinary completeness, and HPLC has slowly replaced colorimetry as the main instrument. The method by Jakobsen *et al* is the most frequently adopted method [8]; however, the described HPLC mobile phase has little buffer capacity at the targeted pH of 3.5. The best buffer capacity occurs at a pH value close to the pK_A of the buffer, and the pK_A values of the phosphate buffer used by Jakobsen *et al*, 2.1, 7.2 and 12.3, are too far removed from 3.5. The buffer concentration was also unusually low (20 mM). The proportions of the different ionic forms of PABA – and thus polarity and retention behavior – are very pH sensitive. Therefore the mobile phase used in this study was changed to consist of 8.4% acetonitrile and 91.6% of a citric acid - phosphate buffer. Citrate has pK_A values at 3.1, 4.8 and 6.4 and is thus an appropriate buffer at pH 3.5. Buffer strength was further increased by using higher concentrations of citrate (100 mM)

and phosphate (200 mM). This led to more reproducible retention times than with the original method.

This study further optimized the alkaline hydrolysis reaction used for the PABA analysis.

PABA Analysis – Alkaline Hydrolysis

The hydrolysis of PABA metabolites to PABA has been studied extensively. The hydrolysis used for the colorimetric method was first developed using HCl as a reactant. However, Tetlow *et al* later replaced HCl with NaOH, because NaOH was shown to give better results during PABA quantification with the colorimetric method [59]. A similar result was also found in this study. NaOH was more suitable for the hydrolysis of PABA for the HPLC method, with the chromatograms showing sharper and more symmetrical peaks than when HCl was used (Figure 12).

The conversion of PABA metabolites back to PABA through alkaline hydrolysis requires heating; however the heating apparatus was not specified by Jakobsen *et al* [8]. The described temperature of 121 ºC makes it likely that an autoclave was used. For this thesis the effectiveness of a microwave, a boiling water bath and an autoclave were investigated. Microwave ovens have been increasingly used during organic reactions as they are convenient and could greatly reduce the time required for the reaction [68]. Nevertheless, the household microwave in this study was found to be associated with

high imprecision and high inter-run variation (Figure 13). Therefore, this heating method was undesirable for the PABA alkaline hydrolysis.

A boiling water bath has been frequently employed for heating the alkaline hydrolysis of PABA metabolites in the past [7, 69, 70]. However, in those studies, the colorimetric method was used for the analysis of PABA. The colorimetric method detects aromatic amines, therefore both PABA and PAH will react. This means that complete hydrolysis of PABA is not required. However unlike the colorimetric method, the HPLC method detects PABA and PAH separately. The HPLC method by Jakobsen *et al* [8] measures only PABA, and therefore requires the complete conversion of all the metabolites back to PABA. The hydrolysis time profile of the PAAB, PAH and PAHA and the degradation time profile of PABA were investigated. It was found that PAHA converts back to PABA through an intermediate PAH, as described by previous literature [69]. It was also found that despite prolonged heating, complete conversion of all metabolites was not achieved (Figure 14). It is likely that a temperature of 100℃ is not sufficient to provide the required reaction energy for the conversion of metabolites back to PABA. Based on the hydrolysis profile of the metabolites, the water bath experimental procedures and the HPLC analysis method were modified to include the quantification of metabolite PAH for the calculation of PABA recovery. The inclusion of PAH of the modified water bath method significantly altered the PABA recovery and the outcome of participants' compliance (Table 2). Compared to the hydrolysis reaction procedure

described by Jakobsen *et al*, this modified hydrolysis reaction heated with a boiling water bath has the advantage of shorter reaction time and a more readily accessible instrument than an autoclave. However, the incomplete hydrolysis makes inclusion of PABA and PAH simultaneously into the calculation of PABA recovery necessary, which complicates the data reduction process somewhat. It could be argued that no hydrolysis is necessary at all, if PABA and all three major metabolites were quantitated simultaneously by HPLC. The disadvantage of this approach is that calibration and quality control of the analysis would become more involved and imprecision may be increased. Run times would also have to be extended to ensure complete elution of all metabolites. Furthermore, minor hydrolysable metabolites other than PAH, PAHA and PAAB would escape the analysis. Therefore the water bath method, described here for the first time, combines PABA and only one metabolite, PAH, as a compromise. This allows analysis of PABA recovery when an autoclave is unavailable, without adding undue resource requirements for larger studies.

The autoclaved alkaline hydrolysis reaction was used to achieve the specified 121℃. However, contrary to the previous literature [8], the conversion of the PABA metabolite PAHA back to PABA was not found to be complete after 90 minutes of alkaline hydrolysis. Jakobsen *et al* did not report any difficulty for the hydrolysis reaction besides that the reaction time was prolonged to 90 minutes to increase the reaction yield of PAH [8]. The hydrolysis reaction of the more resistant PAHA was not studied by them.

Nevertheless, it was found in this thesis that the hydrolysis of PAHA to PABA had a low conversion yield of 51%. A low reaction yield for PAHA of 12% with heating at 120 ℃ for 60 minutes was shown previously by Ito *et al* [71]. By increasing the reaction time to 120 minutes complete conversion of PABA metabolites to PABA was possible. The results obtained from the water bath method, when both PAH and PABA were included in the calculation, were found to be statistically similar to those of the autoclave method. Because the required calculations could not easily be automated with the available chromatography software, the autoclave method was deemed suitable for this large scale analysis.

Although Jakobsen *et al* [8] did not describe heating of calibrators along with urine samples, the study at hand found that CVs could be improved from over 10% to less than 4% when both calibrators and unknown samples were autoclaved to compensate for variation due to the hydrolysis (Table 7).

The method by Jakobsen *et al* [8] uses 25 mL Erlenmeyer flasks for the hydrolysis, glassware that is no longer easily available in modern special chemistry hospital laboratories. The current work used urine volumes of 20 µL instead of 1mL, allowing the use of disposable 2 mL autosampler vials instead of 25 mL flasks. This simplifies the sample preparation considerably by eliminating a transfer step. Larger sample batches can be processed more easily due to lower space requirements and no washing of glassware is necessary, which saves time and reduces the risk of

contamination and carryover. Furthermore, the smaller sample requirement allows re-analysis of volume limited specimens.

In short, PABA analysis by HPLC was optimized and validated for the use in large-scale studies.

C. Completeness of 24-Hour Urine Collection

Urinary Completeness Based on PABA Recovery

The mean PABA recovery was 88.4 ± 15.8%, which is comparable to the previously reported value of 87.9 by Jakobsen *et al* [8]. Jakobsen *et al* previously demonstrated that the PABA excretion decreased with age, and an advanced dosage schedule could not overcome this decrease [72]. Therefore, having an older population could, in theory, lower the mean PABA recovery of that population. However, when the participants were stratified into three age groups, 37-53 years (n = 239), 54-59 years (n = 224), and 59-66 years (n = 218) (Table 14), no statistically significant difference in PABA recovery was observed between participants in different age groups. Furthermore, the inverse relationship between PABA recovery and age was not found to be significant, contrary to the findings from previous literature [8, 72, 73]. That discrepancy was most likely due to the fact that for the current study participants aged over 66 years were excluded from the PABA analysis. Based on the study by Leclercq *et al*, compared to people aged 69 and under, people over 70 years of age were found to have lower PABA excretion [73]. By excluding participants older than 70 years of age, the current study had

excluded data that could potentially make the association between age and PABA recovery significant. The reason for lower PABA recovery in older age is assumed to be reduced renal clearance [73], but bioavailability or increased formation of metabolites that cannot be hydrolyzed to PABA may play a role as well [73]. Age is only a surrogate for decreased PABA excretion. More desirable would be personalized assessments of the pharmacokinetic parameters absorption, distribution, metabolism and elimination, to avoid false qualifications and disqualifications of study participants for PABA eligibility. Until the underlying mechanisms of reduced PABA recovery are understood better, the increased cost and inconvenience of such additional examinations appear not justified. Therefore exclusion from PABA based on age may still be the best compromise.

Among 681 participants included in this study, 64.3% of the participants had completed urine collection with PABA recovery between 85-110%. The proportion of completed samples was found to be greater than other large-scale population studies that also used PABA for validating urinary completeness [74-77]. The higher completion rate was likely due to higher participants' motivation. When the participants' self-reported assessment was used, the proportion of the completed urine collection increased to 72.8%. Reporting bias could possibly contribute to this increase in the amount of completed urine samples.

Sources of PABA include multivitamins, vitamin B complex, and food items such as mushrooms, eggs and milk. It was found that the dietary sources of PABA, when no

vitamin supplements were taken, can range from 6-30 mg/day (12.5% of administered dose) [7, 78]. However, since the colorimetric method was used for the quantification, the dietary PABA reported in these studies could be an over-estimation of the true amount. In this study, no PABA was found in the random draw of blank urines, so the dietary sources of PABA should be negligible. On the other hand, brand name vitamin supplements can contain up to 75 mg of PABA (APPENDIX A). It is therefore necessary to account for the brand, type and amount of the supplements that the participants have taken. Although the vitamin intake information was collected in this study, no information about PABA content in the vitamins was included. Therefore, it would be difficult to know how many samples were misclassified as completed collections due to ingestion of PABA containing supplements.

Another disadvantage of PABA validation is the requirement of post-recruitment screening. First of all, because only people aged below 66 years are qualified to take PABA, it cannot be used for studies that need to evaluate the older population. Besides the age limit, the study participants were screened again, after urine collection had been complete, based on the last PABA pill ingestion time. In this study, roughly 44% of the participants were excluded because they had delayed PABA ingestion which, resulted in a lower PABA recovery regardless of the urinary completeness. Therefore, the urine and data collected from them could not be used, and thus resulted in wasted efforts and

expenses. In the end, only 53% of the enrolled participants were included for further analysis (Figure 7).

Urinary Completeness Based on Self-reported Assessment

For the determination of urinary completeness, participants' responses to two questions were used: Question 1, "During the 24-hour collection period, did you always void into the urine collection container," and Question 2, "Is any urine missing from the collection for any other reason." The participants' responses were used independently, in combination with the AND and OR rules, and then the resulting completeness data were compared to the PABA recovery results. The overall agreement between the results of self-reporting and PABA recovery was slightly above 60%. When Question 1 was used by itself, the test was highly sensitive; however, the test was not statistically significant (Table 9). A similar observation was made when the OR rule was used (Table 12). When Question 2 was used independently, the test was found to be statistically significant, with a sensitivity of 77% and a specificity of 32% (Table 10). Similarly, when the AND rule was used, the test was statistically significant with a sensitivity of 76% and a specificity of 33% (Table 11). The overall agreement with PABA completeness results and the effectiveness of both of these tests were similar.

In addition, average sodium excretions of participants' with complete collections were found to be statistically different from those with incomplete collections, when PABA recovery, Question 2 and the AND rule were used for determining urinary

collection completeness (Table 15). This suggests that Question 2 and the AND rule may have similar effectiveness as PABA recovery for validating completeness. Unadjusted sodium excretion was used for this part of the analysis because the adjusted sodium intake was the product of PABA validation and would not be available if only self-report was used. Furthermore, the average sodium excretion was found to be similar when different completeness criteria were used. Nevertheless, using Question 2 by itself may be more suitable than using the AND rule because only one question would be needed. Having two questions on the questionnaire may cause potential confusion, where participants could go back and change the answer to Question 1 after reading Question 2.

Using the participants' self-reported responses to Question 2 by itself can most effectively determine the completeness of 24-hour urine compared to the other combinations of Question 1 and 2 described above, although according to kappa statistics there was only poor agreement slightly exceeding chance between Question 2 and PABA recovery for determining urine completeness. However, because the mean sodium excretion was found to be similar among those who had completed urine determined by Question 2 and PABA validation (3,577 mg/day vs. 3,643 mg/day), the choice of validation method had little influence on the sodium level of the PURE24USE study. Subar *et al* also found similar levels of protein and potassium within urine samples that were considered complete by PABA validation and participants' self-report [79].

Furthermore, the self-reported assessment was found to be more effective compared to the creatinine validation method. The 69% sensitivity and 23% specificity of the creatinine validation were worse than those of the Question 2 (77% sensitivity, 32% specificity) [55] (APPENDIX B). Among those with completed collection deemed by creatinine, the mean sodium excretion was 3,446 ± 1,539 mg/day, which differed from that of both PABA and Question 2 validation.

In conclusion, self-reported assessment is more effective than creatinine validation, much less cumbersome than PABA validation and lead to similar sodium results in the PURE24USE study. Nevertheless, the agreement was not good enough to consider self-report equivalent to PABA analysis, whenever the strictest criteria of 24-hour urine completeness need to be applied.

Measures of accuracy of the questions to detect urine completeness are not referenced to a diagnostic gold standard, such as urine collection from highly reliable or observed individuals. Instead, PABA was taken as the best available surrogate for true urine completeness. To compare the accuracy of the diagnosis of urine completeness by PABA with the questionnaire, a third, higher reference method would have to be used. If PABA itself is the reference method, its accuracy is 100% by definition. The study at hand cannot decide whether results of PABA or of the questions are closer to the truth. It appears safer to assume that the objective marker PABA is more accurate than self-report until proven otherwise.

D. Sodium Intake

The sodium intake was estimated from the adjusted sodium excretion, which included the sodium excretion values of participants with incomplete 24-hour urine collection by using an adjustment equation [74-76, 79]. The measured and adjusted sodium excretions were significantly different for participants with incomplete collection. In addition, the adjusted sodium excretion for incomplete collections was not significantly different from the measured sodium excretion for complete collections. This supports the assumption that the adjustment equation is an appropriate model to extrapolate from incomplete samples to 24-hour excretion values.

The mean sodium intake was found to be 3,673 ± 1,637 mg/day, which was significantly greater than the estimated sodium intake of 3,100 mg/day previously reported by Canadian Community Health Survey (CCHS) in 2004 [67]. However, the sodium intake value reported by CCHS was subject to under-reporting, because 24-hour dietary recall was used for the estimation of daily sodium intake. Garriguet found that under-reporting of energy intake was 9.6% [80]. Furthermore, 24-hour dietary recall did not include the salt added in cooking and at the table, which usually accounts for 10-20% of the dietary sodium [81]. Therefore, it was possible that the actual 2004 Canadian mean sodium intake was around 3,500 mg/day rather than 3,100 mg/day.

The sodium intake found in this study was greater than the previously reported value, despite the ongoing sodium reduction campaign. This apparent increase in sodium

intake is likely due to lack of efforts to reduce intake, and increased consumption of processed food among Canadians. It was found that about 41% of respondents felt that they were eating too much salt in a 2010 public opinion survey [82]. However, the 2008 Tracking Nutrition Trends survey showed that only 12% of the respondents tried to reduce their salt intake [83]. In addition, there was little increase in money spent on processed food from 53% to 54% from 2001 to 2011 [84], and the percent of food consumed outside the home decreased from 30% to 28% from 2001 [85] to 2012 [86]. Thus, the salt intake among Canadians has probably not decreased significantly during the 21th century.

The mean sodium intake was found to be significantly higher for men (4,210 mg/day) than women (3,139 mg/day) (Table 16), before and after taking age and BMI into consideration. In addition, the sodium intake was lower for the older age group, but the difference was not statistically significant, despite the fact that the Tracking Nutrition Trends survey showed that the older population was more aware of the sodium content in food than the younger population [83]. The sodium intake was significantly lower for the older age groups (age 31-50 and age 51-70) in the previous Canadian population study [67].

E. Implications

The PABA quantification method described here has improved HPLC conditions and specifies missing experimental conditions for hydrolysis in the paper by Jakobsen *et*

al [8]. The use of small sample volumes makes it suitable for large epidemiological studies. Furthermore, this method was optimized and validated in the sub-set of the PURE24USE study population, allowing it to be used directly in future epidemiological studies.

This study provides a snapshot of the current sodium intake status in Canada. The mean sodium intake found in this study is significantly greater than the current recommended sodium intake of 2,300 mg/day, with 79% of participants having a sodium intake above the recommended level. Sodium intake seems to have increased over the past decade. This clearly demonstrates that salt reduction campaigns have not achieved the desired effect. Alternative measures must be taken in the future, if the need to lower salt intake remains crucial for improving the population's health.

F. Future Directions

This thesis demonstrated that the participants' self-report on the urinary collection completeness was not worse than PABA recovery in the sense that similar results of sodium excretion were found. Nevertheless, self-report had only moderate agreement with PABA. Because the study participants received two sets of collection completeness checks, a PABA marker and the questionnaire, it was possible that the advantages and disadvantages associated with PABA masked the true effectiveness of the self-report. Participant recall is much more commonly used, more convenient and leads to less exclusions than PABA analysis. Therefore, it would be important to evaluate the

performance of the self-report in an independent setting. This could be achieved by using a third method with even higher reliability than PABA, such as direct observation or housing of participants in an environment without bathrooms to prevent any possible losses of urine. The latter approach was taken in the original validation of PABA. Such a study would be very difficult to perform with hundreds or thousands of participants. Even in a small study such an artificial setting may lead to much more accurate self-report than under realistic conditions, so it may never be possible to assess the true accuracy of recall. It would be possible to re-evaluate PABA against a higher reference method in a small study. The original clinical validations used colorimetric methods of PABA analysis. Contemporary methods such as chromatography are analytically more specific, but require much harsher, carefully validated conditions for the hydrolysis of metabolites, a fact that is rarely acknowledged in the literature on 24-hour urine completeness. The approaches taken in this thesis, complete hydrolysis in an autoclave, or partial hydrolysis in a boiling waterbath with the analysis of PABA + PAH, provide the basis for such a PABA revalidation. Until it can be proven that recall is sufficiently equivalent to PABA, PABA remains the gold standard for completeness as an objective measure.

Because this study was not designed to assess the PABA recovery of complete 24-hour urine collection, the 85-110% limit was adopted in this study. This limit was defined by Bingham and Cummings [7] based on the colorimetric methods. This completion criterion was used instead of the 78% limit used for the HPLC method

because this modified HPLC method employs a complete hydrolysis reaction while the original HPLC method had an incomplete hydrolysis reaction [8]. Therefore, the 78% limit may cause the inclusion of incomplete samples, and the cutoff for the colorimetric method, which produced 10% higher results than the HPLC method, was deemed to be more suitable for the current study [8]. However, it would be useful to assess a completion limit specific for this modified HPLC method to be used in future studies to avoid possible misclassification of the urine samples.

For urine samples with a PABA recovery between 70-84%, an equation was used in this study for adjusting sodium levels:

> Adjusted 24-hour sodium excretion = measured sodium excretion X (93 / % PABA recovery) [66].

This equation appears in some epidemiological studies [74-76, 79], and is back referenced to the paper by Johansson *et al* [66]. However, the relationship described by the original paper by Johansson *et al* was actually the following:

> Adjusted 24-hour sodium excretion = measured sodium excretion (mmol/day) + 0.82(93 - % PABA recovery).

In addition, the second adjustment equation was derived from colorimetric methods, while PURE24USE and the above studies employ the first equation and use HPLC for PABA analysis. It would therefore be useful to establish a relationship between sodium

and PABA recovery that is specific for the HPLC methods to eliminate potential uncertainties.

For future studies sodium intake is best estimated from 24-hour sodium excretion. The urinary completeness should be ensured by using PABA. Despite the downsides associated with PABA validation, it is the recognized standard. Dietary supplements can contain significant amounts of PABA. This creates a risk for paradoxical over-recovery or false positive compliance assessments. Therefore, the brand, dosage, and frequency of vitamin supplements ingested by participants should be recorded.

G. Concluding Remark

It is firmly established that in order to treat CVD, it is important for the patients to lower their blood pressure, which can be effectively achieved by decreasing daily sodium intake. However, researchers have been struggling to find sufficient and irrevocable evidence to support the reverse, where extremely low sodium intake is associated with low blood pressure and low risk for CVD. One of the problems attributed to this conundrum is the estimation of daily sodium intake. The best surrogate for daily sodium intake is 24-hour urinary sodium excretion, which requires PABA for validating the completeness. The cost and the limitations associated with PABA validation render it unfavorable among researchers. This study employed both self-report of urinary completeness and PABA analysis. Self-report led to similar results in sodium excretion but was found to agree poorly with PABA. A simplified and analytically improved

V. DISCUSSION AND CONCLUSION

PABA HPLC quantification method with carefully optimized hydrolysis conditions was established. This will allow more convenient and reliable assessment of the completeness of 24-hour urine collections in the future.

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APPENDIX

APPENDIX A List of Supplements Containing PABA

Brand	Product – PABA Content
Webber	Multisure for women – 7.5 mg B100 Complex – excipient Multi Vitamin with B Vitamins and Minerals Tablets – excipient
Jamiesen	B100 Complex – 10 mg Timed Release B Complex – excipient VITAMINS B Complex with Vitamin C – 15 mg
Swiss	B Complex with Vitamin C – 15 mg Swiss One 50 Multi Vitamin & Mineral Timed Release – 25 mg Swiss One 80 Multi Vitamin & Mineral Timed Release – 75 mg

APPENDIX B Creatinine Check for Completeness of 24-hour Urine Collection

Table 17: Completeness of urine collection based on PABA recovery and creatinine level.

		Complete 24hour urine collection (PABA 85-110)		
		Yes	NO	Total
24-hour urine completeness based on creatinine	Complete	304	188	492
		61.8%	38.2%	100.0%
	Incomplete	134	55	189
		70.9%	29.1%	100.0%
Total		438	243	681
		64.3%	35.7%	100.0%

Urinary creatinine was also measured and recorded in the PURE24USE study. When using creatinine to validate the completeness of 24-hour urine collection, samples with creatinine level outside the reference range were considered as incomplete: male 14-26 mg/kg/day and female 11-20 mg/kg/day [55]. By applying the creatinine check and comparing it to PABA recovery, the creatinine check had a sensitivity of 69% and specificity of 23%. Overall agreement with the PABA recovery was 53%. In addition, the average sodium excretion was 3,446 ± 1,539 mg/day among participants with completed urine determined by creatinine and 3,552 ± 1,602 mg/day for incomplete collection. The average sodium excretion of the complete and incomplete collection were not statistically different, $t(679) = 0.794$, $p = 0.427$.