THE PHARMACOKINETIC OF SILVER DIAMINE FLUORIDE IN HEALTHY CHILDREN WITH DENTAL CARIES

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FACULTY OF DENTISTRY
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KUALA LUMPUR

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THE PHARMACOKINETIC OF SILVER DIAMINE FLUORIDE IN CHILDREN WITH DENTAL CARIES

ABSTRACT

Silver diamine fluoride (SDF) is a medication used topically on carious tooth surfaces to arrest caries progression in both child and adult populations. This study aimed to determine the pharmacokinetic of SDF in 15 children who received SDF treatment. Urine samples were collected at baseline, and at first and second 24 hours after SDF treatment to determine silver and fluoride levels. Hair samples were collected at baseline, and at days 7, 14, 30, 60, 75, and 90 after SDF to assess silver levels. The median and interquartile range of urinary fluoride at baseline, first and second 24 hours after SDF were 498.33 (381) µg/24-hour, 574.92 (318.87) µg/24-hour, and 474.35 (324.21) µg/24-hour, respectively. The pairwise comparison results indicated that urinary fluoride levels at the first 24 hours after SDF treatment were significantly higher than those at the baseline. However, the higher urinary fluoride levels are not sufficient to cause fluorosis as SDF is not for daily application. The range of urinary silver levels at baseline, and the first and second 24 hours after SDF were <1 µg/24-hour, 1 – 2 µg/24-hour and <1 µg/24-hour, respectively. Although urinary silver levels were higher in the first 24 hours following SDF, statistical analysis was not possible due to their extremely low levels. The range of hair silver levels after SDF treatment (< 0.2-0.69 µg/g) were found to fluctuate around the baseline level (<0.2-1.7 µg/g). Based on these study results, the use of SDF is safe in children.

Keywords: Silver diamine fluoride, caries teeth, children, pharmacokinetic, safety

KAJIAN FARMAKOKINETIK FLUORIDA DIAMINA PERAK DI KALANGAN KANAK-KANAK DENGAN KARIES GIGI

ABSTRAK

Fluorida diamina perak (SDF) merupakan ubat yang diaplikasikan secara terus pada permukaan gigi yang rosak untuk menahan perkembangan karies di dalam kalangan populasi kanak-kanak dan dewasa. Kajian ini bertujuan untuk menentukan farmakokinetik SDF pada 15 orang kanak-kanak yang menerima rawatan SDF. Sampel air kencing telah dikumpul pada garis dasar, 24-jam pertama, dan 24-jam kedua selepas rawatan SDF untuk menentukan tahap perak dan fluorida. Sampel rambut juga dikumpul pada garis dasar, hari 7, 14, 30, 60, 75, dan 90 selepas SDF untuk menilai tahap perak. Median dan julat antara kuartil fluorida dalam air kencing pada garis dasar, 24-jam pertama dan 24-jam kedua selepas SDF adalah masing-masing 498.33 (381) µg/24-jam, 574.92 (318.87) µg/24-jam dan 474.35 (324.21) µg/24-jam. Keputusan perbandingan berpasangan menunjukkan bahawa tahap fluorida dalam air kencing pada 24-jam pertama selepas rawatan SDF adalah ketara lebih tinggi dari tahap fluorida air kencing garis dasar. Walau bagaimanapun, tahap fluorida yang lebih tinggi ini tidak mencukupi untuk menyebabkan fluorosis kerana SDF bukan untuk aplikasi harian. Julat tahap perak dalam air kencing pada garis dasar, 24-jam pertama dan 24-jam kedua selepas SDF adalah masing-masing <1 µg/24-jam, 1 – 2 µg/24-jam and <1 µg/24-jam. Walaupun tahap perak dalam air kencing adalah lebih tinggi dalam masa 24-jam selepas SDF, namun analisis statistik tidak dapat dilakukan kerana tahap perak dalam air kencing adalah terlalu rendah. Julat tahap perak pada rambut selepas rawatan SDF (<0.2-0.69µg/g) didapati turun naik di sekitar garis dasar (<0.2-1.7 µg/g). Berdasarkan dapatan kajian ini, penggunaan SDF dalam kalangan kanak-kanak adalah selamat.

Kata kunci: Fluorida diamin perak, karies gigi, kanak-kanak, farmakokinetik, keselamatan

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LIST OF SYMBOLS AND ABBREVIATIONS

ALS	:	Australian Laboratory Services
ATSDR	:	Agency for Toxic Substances and Disease Registry
BMI	:	Body mass index
CDC	:	Centers for Disease Control and Prevention
C_{max}	:	Highest concentration of a drug in the blood, cerebrospinal fluid, or target organ after a dose is given
CTQ	:	Centre de Toxicologie du Québec
F1	:	Urinary fluoride level in the first 24 hours following SDF treatment
F2	:	Urinary fluoride level in the second 24 hours following SDF treatment
ICP-MS	:	Inductively coupled plasma-mass spectrometry
IQC	:	Internal quality control
ISE	:	Ion selective electrode
IUPAC	:	International Union of Pure and Applied Chemistry
LOD	:	Limit of detection or method detection limit
LOQ	:	Limit of quantification
SDF	:	Silver diamine fluoride
ppm	:	Parts per million
MOH	:	Ministry of Health Malaysia
NIH	:	National Institutes of Health Malaysia

NIOSH	:	National Institute of Occupational Safety & Health
PK	:	Pharmacokinetic
RfD	:	Oral reference dose
RSD	:	Relative Standard Deviation
SD	:	Standard deviation
T_{max}	:	The time to reach C_{max}
$T_{1/2}$	:	time taken for C_{max} to drop in half (half-life)
U.S EPA	:	United States Environmental Protection Agency
U.S FDA	:	United States Food and Drug Administration
WHO	:	World Health Organization

CHAPTER 1: INTRODUCTION

Dental caries is a biofilm-mediated, diet-modulated, multifactorial, non-communicable dynamic disease resulting in net mineral loss of dental hard tissues (Pitts et al., 2017). Oral diseases affect half the world's population of approximately 3.58 billion people (James et al., 2018). Traditionally, the treatment of dental caries involves their removal using dental drills and restoration. Contemporarily, caries is managed in a minimally invasive way (Mm et al., 2014). Instead of surgical treatment, minimal invasive dentistry (MID) focuses on caries arrest procedures. Silver diamine fluoride (SDF) as part of MID can effectively arrest cavitated caries lesions (IAPD, 2020).

SDF is a colorless and odorless solution at alkaline pH (Mei et al., 2014). Its main components are silver, fluoride, and ammonia (Yamaga et al., 1972). Silver is an antimicrobial agent. The ammonia stabilizes the solution, while the fluoride aids remineralization (Yamaga et al., 1972). The concentrations of SDF in the market are 12, 30, and 38% SDF. The most used concentration is 38% (Chu et al., 2002; Llodra et al., 2005). A 38% SDF contains 24.4 - 28.8% (253,870 ppm) volume of silver, 5.0 - 5.9% fluoride (44,800 ppm), and ammonia (Mei et al., 2014). The ammonia acts as a stabilizing agent for the SDF solution. In Malaysia, the only available SDF is Riva Star®, which is a 38% SDF.

The recommended clinical application of SDF (Advantage Arrest®) is one drop per 10-kilogram bodyweight per treatment visit (Horst et al., 2016) biannually (Seifo et al., 2020). The manufacturer of Riva Star® recommends using one capsule of SDF per treatment visit, and a second application may be administered after one week. Although the amount of SDF applied is minute, clinicians should avoid multiple and frequent applications on young children. At present, the safety of SDF use is not indisputable (Chu & Lo, 2008; Gotjamanos & Afonso, 1997; Gotjamanos & Orton, 1998).

The toxicity of SDF is related to the silver and fluoride compounds. The critical long-term effect of ingesting silver is argyria, a medically benign but permanent bluish-grey discoloration of the skin (Lansdown, 2010). On the other hand, the untoward effect of the use of fluoride is enamel fluorosis, a developmental dental defect characterised by enamel with lower mineral content and increased porosity secondary to increased fluoride exposure during dental development (DenBesten, & Li, 2011).

Blood, urine, and hair are the common biomarkers of exposure to silver and fluoride. Research involving minors should prioritize risk no greater than minimal (European Union, 2008; National Institutes of Health Malaysia, 2011). Minimal risk is defined as the probability and magnitude of physical or psychological harm generally encountered in daily life or during routine medical, dental, or psychological examinations of healthy persons (National Institutes of Health Malaysia, 2011). Blood sampling is not considered regular dental care and thus is not the chosen biomarker in child populations.

From the literature search, the clinical pharmacokinetic study by (Lin et al., 2019) showed that topical applications of 38% SDF is safe and well-tolerated in healthy adults. In this predecessor study, silver and fluoride levels were measured from urine and blood samples of patients who received SDF treatment. Due to the differences in physiological processes between children and adults, the pharmacokinetic characteristics of SDF in healthy children may differ from healthy adults (Batchelor & Marriott, 2015). Interestingly, according to a recent pharmacokinetic model simulation in children, silver levels in blood and tissue are lower than toxic concentrations (Chen et al., 2020). This simulation model was based on the data in (Lin et al., 2019).

Besides having the highest concentration of fluoride levels among dental products, SDF contains the heavy metal silver. Silver and fluoride's potentially harmful side effects need to be considered when providing SDF treatment, especially for paediatric patients.

The clinicians need to know the amount of silver diamine fluoride applied onto the tooth surfaces in children and whether they are within safe limits.

This thesis is based on a clinical question regarding two issues. First, the amounts of SDF applied to the carious teeth of patients differed. Moreover, the droplet sizes may not be consistent for each use (Crystal et al., 2019). Hence, the recommendation of a drop of SDF per 10-kilogram body weight per visit or a capsule of Riva Star® per visit seems to be a rough estimate. Second, although there are pharmacokinetic simulation models for SDF in children (Chen et al., 2020), they are based on those for adults, and the data generated is often associated with prediction errors and uncertainty (Khalil & Läer, 2011).

This study aims to determine the actual silver and fluoride levels in children receiving SDF treatment. Due to the obvious ethical concerns of drawing blood in children solely for research purposes, urine and hair samples were chosen as the exposure biomarkers. Currently, and to the author's knowledge, based on the literature search, no pharmacokinetic studies have been conducted on silver diamine fluoride using biological samples from children.

1.1. Problem Statement, Research Question and Hypothesis

1.1.1. Problem Statement

There is a lack of data on the amount of SDF applied to children's teeth, the safety of SDF, and the PK characteristics of SDF among child populations.

1.1.2. Research Question

What are the pharmacokinetic characteristics of silver diamine fluoride in terms of silver levels in urine and hair samples and urine samples' fluoride levels among children with dental caries? Any adverse effects following SDF treatment?

1.1.3. Hypothesis

Null hypothesis: The pharmacokinetic characteristics of SDF measured in their biomarkers taken before and after SDF treatment are the same.

1.2. Aim of the Study

To determine the pharmacokinetic of silver and fluoride following topical applications of SDF in children with dental caries.

1.3. Objectives of the Study

The objectives of this study are:

1. To assess the level of silver (µg/24-hour) in urine samples of children with dental caries and to derive their pharmacokinetic profiles before and after SDF treatment;

2. To assess the level of fluoride (µg/24-hour) in urine samples of children with dental caries and to derive their pharmacokinetic profiles before and after SDF treatment; and

3. To assess the level of silver (µg/g) in hair samples of children with dental caries and its pharmacokinetic profile before and after SDF treatment.

CHAPTER 2: LITERATURE REVIEW

2.1 Dental Caries in Children

Dental caries is a continuously changing process involving repetitious periods of demineralization and subsequent remineralization that generally result in an imbalance in the demineralization of the tooth substance in the oral environment (Dean et al., 2016). The incidence of dental caries is substantial. Globally, it is the most prevalent condition according to a Global Burden of Disease Study in 2015 (Wang et al., 2016). In Malaysia, 71.3% of preschool children have dental caries, of which 63.6% require restoration of deciduous teeth with caries (Salleh et al., 2017).

Early Childhood Caries (ECC) is defined as the presence of one or more decayed (non-cavitated or cavitated lesions), missing or filled (due to caries) surfaces in any deciduous tooth of a child aged below six years ("Early Childhood Caries: IAPD Bangkok Declaration," 2019). There are three phases in the management of ECC. Primary prevention involves:

- Educating parents/caregivers and healthcare workers about oral health knowledge,
- Reducing children's consumption of free sugar in foods and beverages, and
- Daily fluoride exposure.

Secondary prevention is by means of efficient control of early caries lesions before they develop into cavities. Examples include regular topical fluoride varnish applications and pit and fissure sealant treatment of vulnerable molars. Halting the caries process on a tooth with an open cavity and tooth-preserving dental treatment is part of tertiary prevention ("Early Childhood Caries: IAPD Bangkok Declaration," 2019).

There is no consensus on the operative management of dental caries. Based on a scoping review by *Children Experiencing Dental Anxiety: Collaboration on Research and Education* (CEDACORE), treatment modalities can be classified into the treatment of early caries lesions and cavitated lesions. For early caries lesions, monitoring, topical fluoride applications, resin infiltration, pits and fissure sealants were recommended. In a cavitated tooth, the application of silver diamine fluoride, using a non-restorative approach, tooth restoration, and extraction were the treatments of choice (Correa-Faria et al., 2020).

In comparison to other interventions in controlling the progress of caries in primary dentition, SDF is more efficient. The efficacy of SDF has extended to the prevention of caries in the whole dentition as well (Chibinski et al., 2017). Based on evidence from a systematic review, the inherent ability of SDF to arrest both cavitated and non-cavitated caries lesions is high (Schmoeckel et al., 2020). According to a World Health Organization manual for ending early childhood caries, primary care dentists are advised to apply SDF onto caries teeth involving the dentin layer (World Health Organization, 2019a). Single- or twice-yearly SDF applications are highly effective in stopping the progression of active caries in deciduous teeth.

2.2 Silver Diamine Fluoride

2.2.1 The History of Silver Diamine Fluoride

Silver diamine fluoride (SDF) was first produced by Dr. Mizuho Nishino (Nishino et al., 1969), who mixed silver and fluoride to create a new compound, namely silver fluoride. The purpose was to combine the beneficial antibacterial activity attributes of silver and the remineralization effects of fluoride. Caries prevention, arrestment, and desensitization of hypersensitive dentin were the main effects (Nishino et al., 1969). The

penetration of SDF into enamel and dentin were up to 20 and 100 micrometres, respectively, with silver penetrating deeper into the pulp chamber (Yamaga et al., 1972).

In the 1970s, SDF was marketed in Japan under Saforide ® as an agent to halt caries progression. SDF has many names, for example, diammine silver fluoride, silver fluoride, and silver ammonium fluoride. Interesting, the most-used term "silver diamine fluoride" (single m) was a misnomer. The two ammine (NH_3) groups of SDF should be termed "silver diammine fluoride" (Lo & Duangthip, 2019).

The US Food and Drug Administration (FDA) cleared SDF as a medical device for treating tooth sensitivity in 2014. In 2016, SDF (Advantage Arrest®) was awarded breakthrough therapy status by the FDA as a drug to treat severe early childhood caries (press release by Elevate Oral Care, October 30, 2016). Indications and contraindications for the use of SDF and the advantages and disadvantages are described in Table 2.1 (Seifo et al., 2020).

Table 2.1 Indications, contraindications, advantages, and disadvantages of SDF

Use of SDF	Level	Description
Indications	Tooth	Cavitated dentine carious lesions with no symptoms in deciduous teeth
		Cleansable carious lesions
		Dentine lesions that are not restorable
		A few carious lesions that may not all be managed in single visit
		Root caries (deciduous and permanent teeth)
		Non-carious cervical lesions with dentine hypersensitivity
		Sensitive teeth affected by molar incisor hypomineralisation
	Person	Behaviour/ medical conditions of pre-cooperative children and adults which limit invasive restorative treatment and where there is a need to avoid or delay treatment with sedation or general anesthesia.

		Patients with high caries risk with medical or psychological conditions that limit other treatment approaches e.g., patients with dental phobia, medical conditions, or disabilities.
		Patients who already have a high standard of brushing or are likely to be responsive to measures to change behaviour to carry out frequent, high quality toothbrushing or other methods to clean carious lesions.
Contra-indications	Tooth	Clinical signs or symptoms of irreversible pulpitis, or dental abscess/fistula
		Radiographic signs of pulpal involvement, or peri-radicular pathology
		Infection or pain from pulp or food packing (unless tooth shape can be changed to become cleansable)
	Person	Not able or willing to brush and unlikely to. Patients (or parents) unable or unwilling to take responsibility.
		Potassium iodide is contra-indicated in pregnant or breastfeeding women, patients undergoing thyroid gland therapy or on thyroid medication or patients with known allergy to potassium or iodine.
		Patients with ulceration, mucositis, stomatitis
		Patients with allergy to silver, fluoride, or ammonia
Advantages	Tooth	Can help to maintain space in the primary/mixed dentition if avoiding the extraction of a tooth/root.
	Person	May avoid restoration or dental extraction in patients who would find this difficult to cope with (medically or psychologically).
		Useful for improving cooperation in anxious or pre-cooperative individuals by allowing a minimally invasive treatment then building on successful treatment and coping strategies
Disadvantages	Tooth	Not always successful. Relies to some extent on cleansability and cleansing by the patient/caregiver or biofilm profile, and these may not be possible to predict.
		Difficult to monitor success
		Might not work rapidly enough or with enough success to avoid the pulp becoming irreversibly damaged or infected.
	Person	Aesthetics
		Feeling of delaying/avoiding a traditional restorative solution

2.2.2 Mechanism Action of Silver Diamine Fluoride

The mechanism of action of SDF is theorized to be based on the antibacterial and remineralization actions of silver and fluoride, respectively (Zhao et al., 2018). Silver causes bacterial cell death. However, the exact mechanism is unknown, although a few hypotheses have been proposed (Dakal et al., 2016; Lansdown, 2010):

1. Silver ions bind to the cell membranes of microorganisms leading to cell content leakage, disruption of cell motility (in motile bacteria), and cell death.

2. Silver ions appear to be toxic and thus poison the metabolic enzymes and block electron transport systems.

3. Silver ions inactivate the bacterial DNA and RNA via unknown pathways (Lansdown, 2002; Marx & Barillo, 2014).

The anti-microbial effects have been shown against *Streptococcus mutans* and *Actinomyces naeslundii* on dentin surfaces (Chu et al., 2012). Studies suggest that SDF does not allow for biofilms to adhere, thereby reducing the colonization of bacteria on enamel surfaces (Chu et al., 2012; Wu et al., 2007).

The fluoride inhibits demineralization and enhances remineralization on the dental hard tissues (Buzalaf et al., 2011). The fluoride ions in SDF mainly act on enamel hydroxyapatite. The fluorapatite [Ca10(PO4)6F2] that is formed is more impervious to acidic environments (Fung et al., 2013).

Upon application of SDF on carious tooth tissue, SDF desensitizes the tooth and stops caries progression through blockage of the dentin tubule. The carious enamel and dentin are permanently stained black (Fung et al., 2013). The black-stained layer shows a hard and impermeable coating of silver phosphate that protects the exposure to dentin collagen ("Silver Diamine Fluoride: What Is Its Place In Oral Healthcare?, " n.d.).

Histologically, a layer of irregular tertiary dentin with silver is deposited to a depth of 1mm in the dentinal tubules. The absence of bacteria and pulpal inflammation is observed in deep carious primary teeth treated with SDF (Bimstein & Damm, 2018).

2.2.3 Caries Prevention and Arrest by Silver Diamine Fluoride

SDF has caries prevention and arrest properties, although its exact caries prevention mechanism effects are still not known. The high concentration of SDF should not be assumed to be the reason behind its high caries arresting ability (Oliveira et al., 2019). It is hypothesized that following SDF treatment, the silver chloride and silver phosphate layer formed on the tooth surface protect the enamel and dentin. The calcium and phosphorus ions on the tooth surfaces are protected from loss (Zhao et al., 2018).

A recent systematic review and meta-analysis compared SDF with placebo or active treatments in preventing the development of new caries in the primary dentition (Oliveira et al., 2019). Children who had SDF treatment for caries lesions or on all primary teeth were assessed in terms of prevention of new lesions on tooth surfaces against those who did not receive any SDF treatment. SDF was reported to arrest caries significantly better than the placebo (preventive fraction 77.5%) and 5% sodium fluoride varnish (preventive fraction 41.5% and 54% at 18- and 30-month follow-up, respectively). Compared to SDF, glass ionomer restorations performed better, although not statistically significant. This systematic review shows that when SDF is used to arrest caries, its anticaries benefits to the entire primary dentition are high. A 77.5% reduction of new caries development was observed for the SDF compared to the placebo groups (Seifo et al., 2018).

Based on a systematic review, the caries arrest rates of SDF in primary dentition ranged from 65 to 91%, whereas the arrest rates for fluoride varnish, glass ionomer

cement, and placebo ranged from 38 - 44%, 39 - 82%, and 34%, respectively (Duangthip et al., 2015). The SDF used in the included studies were 30 and 38% SDF. These studies reported that topical applications of SDF could stop caries progression in the primary dentition. The evidence of caries arrest effects of SDF in primary dentition is substantial, as systematic reviews consistently report congruent findings (Seifo et al., 2019).

SDF products come with different concentrations of 12, 30, and 38%. The 38% SDF has been studied extensively and found to be superior to the other two in arresting dental caries (Jabin et al., 2020). For primary dentition, 38% SDF is shown to be effective and safe in controlling dental caries based on a systematic review (Jabin et al., 2020). All four studies selected in this systematic review show that 38% SDF is superior for caries arrest compared to other interventions, including the lower SDF concentration of 12% or sodium fluoride varnish (Jabin et al., 2020). Clinical outcomes were measured differently; for example, changes in active caries surface to arrested caries surface (Yee et al., 2009) and an average number of new caries surfaces appearing in primary teeth (Llodra et al., 2005). Regardless of clinical outcome measurement methods, 38% SDF is advocated as the best treatment option to arrest caries in primary teeth. Another systematic review also supports the finding that 38% of SDF consistently show a higher chance of arresting dentin caries in primary teeth than the 12% SDF concentration (Tolba et al., 2019). In terms of frequency, biannual rather than annual applications of 38% SDF is more effective in treating primary teeth dentin caries (Fung et al., 2016; Zhi et al., 2012).

2.2.4 The Safety Profile of SDF

The safety profile of SDF is vital due to the presence of silver ions and extremely high fluoride concentrations of up to 44,800 ppm in the 38% SDF (Chu & Lo, 2008). Before the approval of SDF (Advantage Arrest®) by the FDA, rat, and mouse

studies were conducted to confirm the lethal dose (LD50) of SDF (Horst et al., 2016). Oral and subcutaneous administration was assessed. Mean LD50 by oral and subcutaneous administration was 520 mg/kg and 380 mg/kg, respectively. A single drop of 38% SDF (Advantage Arrest®) contains about 25 μL and 9.5 mg of SDF, which is sufficient to treat five teeth. Based on an example of a child with a low body weight of around 10 kg, the dose would be 9.5 mg/10 kg or 0.95 mg/kg. By taking the lower LD50 dose (subcutaneous administration) of 380 mg/kg, the relative safety margin of using an entire drop on a 10-kg child is 380 mg/kg divided by 0.95 mg/kg dose, a 400-fold safety margin (Horst et al., 2016). The actual dose is much smaller. For example, 2.37 mg was the most significant dose measured to treat three teeth (Vasquez et al., 2012). According to the treatment protocol of the University of California San Francisco, the recommended limit is one drop (25μL) per 10-kg body weight per treatment visit. The frequency of weekly applications is the limit. According to the EPA, 1.142 mg of silver per liter of drinking water for 1-10 days is the allowable short-term exposure limit, while 9.5 mg of SDF per drop is within the permissible limit (U.S Environmental Protection Agency, 2003; ATSDR, 1990).

No cases of acute toxicity with SDF were reported in a survey conducted (Duangthip et al., 2018) and in clinical trials in pre-school and school children (Chu et al., 2002; Llodra et al., 2005). However, it should be noted that for 40% silver fluoride, the high concentration of fluoride could lead to dental fluorosis in young children (Gotjamanos, 1997). This is based on an *in vitro* study that indicating when 40% silver fluoride is used to treat deep caries lesions, 3 to 4 mg of fluoride could enter the systemic circulation. It is due to the use of cotton pledgets when applying silver fluoride resulting in an inconsistent way of measurement as the pledgets came in different sizes and masses, ranging from 2.5-16.6 mg (Gotjamanos, 1997). For SDF, similar issues with measurement exist. The precise quantity of silver and fluoride present in a single drop of

SDF is determined by the specific gravity of the liquid and the dropper used (Crystal et al., 2019).

2.2.5 The Stability of SDF Compound

Three *in vitro studies* had proved that the exact dosage of SDF may vary from the manufacturers' claims (Crystal et al., 2019; Mei et al., 2013; Soares-Yoshikawa et al., 2020). The SDF products involved are Ancárie®, Cariestop®, Saforide® and Advantage Arrest®. Both Ancarie® and Cariestop® are not alkaline, with a pH ranging from 4.5 to 8. It is important for SDF to be alkaline (around pH 10) as this pH helps in stabilizing the solution. The SDF products with an acidic pH may not have beneficial effects in arresting caries (Soares-Yoshikawa et al., 2020). The average fluoride content of Saforide® and Advantage Arrest® were higher than the expected concentrations of 45,283 ppm (Soares-Yoshikawa et al., 2020). (Mei et al., 2013) reported similar findings with Saforide® containing an average of 55,800 ppm fluoride. Besides, they also showed that fluoride concentrations of both 12 and 30% Cariestop® were lower than the advertised concentrations. Some SDF products demonstrated changes of fluoride concentrations after the opening. In the case of Advantage Arrest®, (Crystal et al., 2019) reported that the product delivers the advertised concentration of fluoride when first opened and fluoride concentration became higher than the manufacturer's claims (45,215-51,000ppm) from days 7 to day 28 after opening. At day 0 and day 7 after opening, 40-50% of individual sample values were above the limit described by the manufacturer (51,000ppm), and this increased to 93% at day 28 (54,000 ppm). On the other hand, for Cariestop® and Saforide®, Mei and colleagues had different findings. They reported no significant changes of fluoride concentration for both Cariestop® and Saforide® after day 7 and day 28 of opening. The current evidence showed concerning issue regarding

the different fluoride concentrations of SDF from the manufacturers' claim and the increment of fluoride concentrations after bottle opening.

The silver concentration in SDF products seemed to have similar issues (Crystal et al., 2019) reported that Advantage Arrest® delivered the advertised 256,721 ppm through 289,565 ppm of silver when it's first opened. Still, the concentrations reduced from 7 through 28 days to less than 249,000 ppm on day 28. Table 2.2 summarizes the methods and findings from the studies mentioned above.

Table 2.2 Summary of the studies evaluating fluoride and silver concentrations of SDF

Author, year of study	Studied SDF brand	Methods	Findings
Mei et al., 2013	SDF used, expected fluoride ions concentrations, expected pH: I.Cariestop-12%, 14,150ppm, 8.5 II.Cariestop-30%, 35,400ppm, 8.5 III.Saforide-38%, 44,800ppm, pH not mentioned	The fluoride ion concentrations and the pH were determined. The measurements were performed when open and at 7 and 28 days.	**The mean fluoride ion concentrations of the freshly opened bottles were:** I.Cariestop-12%: 12,525ppm II.Cariestop-30%: 13,200ppm III.Saforide-38%: 55,800ppm. **The mean pH values were:** I. Cariestop-12%: 9.4 II.Cariestop-30%: 10.4 III.Saforide-38%: 10.2 No significant change in the fluoride ion concentrations or the acidity was detected after 7 or 28 days.

Crystal et al., 2019	Advantage Arrest-38% (Fluoride concentrations: 45,215-51,000ppm)	Five samples of 38% SDF were evaluated when the bottle was first opened, and at 7 and 28 days. Fluoride and silver concentrations were determined. pH was measured with a pH probe. Weight and volume of individual drops were measured.	**The fluoride concentrations**: -At days 0 and 7, 40-50% of individual measured values were above the upper limit of expected fluoride concentration (51,000ppm), and at day 28, 93% were above the limit. **The silver concentrations**: -At day 0 and day 7, 13-19% of individual measured values were below the lowest expected silver concentration, and this rate increased to above 93% at day 28 **The pH:** Acidity (pH 10) was consistent over the 3 periods. **The weight and volume of individual drops:** Mean (standard deviation) weight of a drop was 40 (4.0) milligrams, and mean (standard deviation) volume was 32.55 (1.89) microliters, 30% more than the reported value of 25 µL
Soares-Yoshikawa et al., 2020	The products evaluated (expected fluoride concentrations, pH): I- Saforide- 38% (45,283ppm, pH not mentioned) II-Advantage Arrest-38.3 to 43.2% (45,283-	Fluoride concentration in the products was determined and confirmed with microdiffusion. The pH of the products was determined with pH strip.	**The fluoride concentration found in the products (mean ±SD; ppm F):** I-53,491 II-57,249 III-4814 IV-5726 V-10,145 VI-11,858

	51,013ppm, pH not mentioned) III-Ancarie- 12% (14,100ppm, pH 8.5-9) IV-Ancarie- 30% (35,400ppm,pH 8.5-9) V-Cariestop- 12% (14,100ppm, pH 8.5) VI-Cariestop- 30% (35,400ppm, pH 8.5)		**The pH:** I-10 II-10 III-6 IV-4.5 V-8.0 VI-8.0

2.3 Pharmacokinetic Study

The word pharmacokinetic (PK) originates from the Greek *pharmakon* meaning drug, and *kinetikos* meaning movement. It describes the absorption, distribution, metabolism, and excretion of a compound (Cannon, 1996; Turfus et al., 2017). After a drug is orally administered, its concentration in the bloodstream and tissues changes with time, first increasing as it enters the systemic circulation and then decreasing as it is distributed to

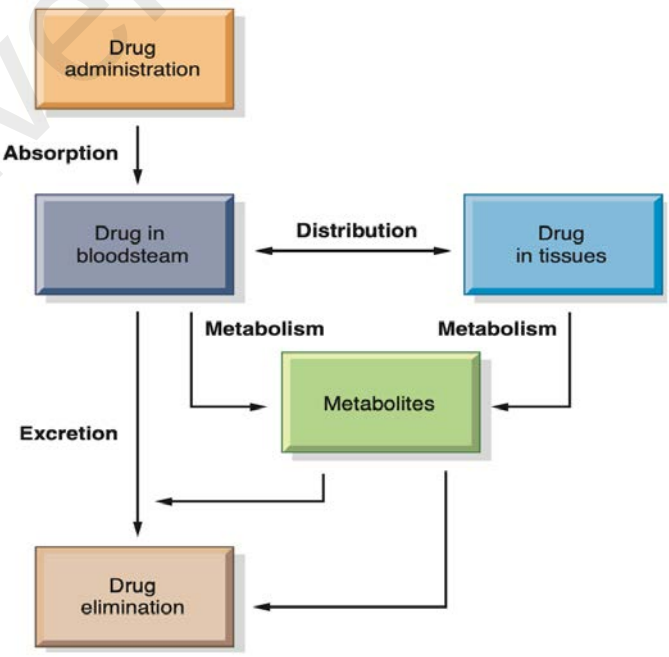

Figure 2.1 Representation of the four elemental pathways of drug movement and modification in the body
Adopted from Sakai (2008)

tissues, metabolized, and excreted. Figure 2.1 illustrates the pathways of drug movement and modification in the body.

Pharmacokinetic describes the time course of the drug and metabolite concentrations in the body. The drug's concentration could be measured in the blood or plasma, urine, saliva, and other easily sampled body fluids/tissues (Spruill et al., 2014). The rate of pharmacokinetic changes relies on physiological factors such as age, ethnicity, or pathological variables (obesity, cardiac impairment) (Ministry of Health Malaysia, 2019).

Clinical studies in pediatric pharmacokinetic are generally undertaken to promote formulation development and determine plasma profile concentrations to support dosing recommendations. Recent regulations have increased the number of clinical trials conducted in children over the last decade. However, there is still a substantial discrepancy between the paediatric burden of disease and the amount of clinical trials research within this population (Batchelor & Marriott, 2015).

The study of PK in children aims to match the exposure in paediatric patients to that found in adults. As the physiological processes between children and adults are different, children should not be regarded as a miniature version of an adult. Conventional designs of pharmacokinetic study cover the following (Sakai, 2008):

1. Administering a standard dosage of the investigated drug, either orally or intravenously.
2. Drawing blood from the subjects at multiple time points to analyze the amount of drug per blood volume at each point.
3. The obtained value represents the drug concentration in blood at specific time points.
4. Collecting a urine sample to assess the appearance of the investigated drug and its metabolites.

5. The drug concentration will rise to a maximum level, then fall off and disappear.
6. Half-life (t1/2): the time it takes for the drug concentration to fall to one-half.
7. The blood concentration of a drug is then used to plot against time to understand its behavior in the body.
8. The data collected from this type of study forms the basis for dosing determination, dosing intervals, and the safety limits of a drug.

Four PK studies on SDF have been performed in adult populations (Lin et al., 2019; Sari et al., 2017; Vasquez et al., 2012; Widianti et al., 2018) with blood used as the biomarker for silver and fluoride levels in two of them (Lin et al., 2019; Vasquez et al., 2012). The study by Vasquez and colleagues measured blood silver and fluoride levels up to four hours after SDF treatment, whereas Lin and colleagues measured the levels up to 24 hours after SDF treatment. These studies reported that fluoride reaches a maximum level at 3 hours and silver within 2.5 - 3 hours (Lin et al., 2019; Vasquez et al., 2012). The authors conclude that SDF is safe and non-toxic when used in the adult population.

Blood sampling for paediatric population trial poses ethical concerns, and alternatives have been used in paediatric clinical studies. These include saliva (Al-Obaidy et al., 1995) and urine sampling (Kole et al., 2011), often preferred by patients and their parents. In the adult population, a saliva study of patients receiving SDF treatment was conducted by Widianti et al. (2018) to analyze the differences in fluoride concentration in saliva before and after silver diamine fluoride application on enamel. Immediately after SDF application on enamel, the fluoride level reached a maximum then returned to the baseline within one hour (Widianti et al., 2018). Sari et al. (2017), who assessed urinary fluoride levels before, and up to three hours, after SDF application, found they peaked three hours after treatment. Both studies also concluded that SDF is safe for use in adults (Sari et al.,

2017). Table 2.3 summarizes these study characteristics and the results of the SDF PK study.

Table 2.3 Characteristics of pharmacokinetic studies of SDF

	Lin et al. (2019)	Vasquez et al. (2012)	Sari et al. (2017)	Widianti et al. (2018)
Subjects	16 adults	6 adults	4 adults	4 adults
Biomarker	Serum, urine	Blood	Urine	Saliva
Maximum serum concentrations for fluoride (C_{max})	Not determined due to serum concentrations near the detection limit of the assay for fluoride	1.86 (μmol/L)	Not studied	Not studied
Maximum salivary fluoride concentrations (C_{max})	Not studied	Not studied	Not studied	7.041ppm
Time when maximum fluoride levels reached (T_{max})	Not determined due to serum concentrations near the detection limit of the assay for fluoride	3 hours	3 hours	Immediately after SDF application
Maximum serum concentrations for silver (C_{max})	0.67±0.49 ng/mL	206 (nmol/L)	Not studied	Not studied
Time when maximum silver levels reached (T_{max})	Median3 hours	2.5 hours	Not studied	Not studied
Urine Fluoride	1.29 ± 0.81 mg	Not studied	0.2160 ppm (mean)	Not studied
Urine Silver	No silver was recovered in the urine	Not studied	Not studied	Not studied

Besides blood, urine, and saliva samples, hair can provide PK parameters (Ngara et al., 2020; Roberts et al., 2015). It has been suggested from the hair bulb blood supply that most external substances or their metabolites passively diffuse into growing hair cells at the follicle base and then bind into the hair shaft (Boumba et al., 2006). The scalp hair

fiber grows at an average rate of 1 cm per month (Cooper et al., 2012). Drug amounts in hair constantly advance until the next haircut or when all the drugs are removed from systemic circulation. Therefore, analyzing hair samples can obtain additional information about the patient's drug exposure over time. The determination of hair PK could predict the amount of drug exposure in plasma (Ngara et al., 2020).

A PK study of silver diamine fluoride in children's populations was published recently. Using physiologically based PK modelling, the simulation suggests that conventional SDF applications to teeth result in plasma and tissue silver concentrations below toxic concentrations. This PK modelling was based on the data of a predecessor study by Lin et al. (2019) which evaluated PK characteristics of SDF in adults (Chen et al., 2020). Generally, physiologically based pharmacokinetic (PBPK) models are based on mathematical concepts in describing physiological, physicochemical, and biochemical processes as they affect the PK characteristics of a drug. PBPK modeling is useful in helping to optimize the conductance of clinical trials in special populations such as paediatric, where optimal planning is required to minimize ethical and technical difficulties (Khalil & Läer, 2011). However, PBPK models do have limitations and weaknesses. For instance, some physiological processes are poorly characterized because they reflect current scientific knowledge, and information gaps may exist. Besides, the validity of the simulations depends on the corresponding model and its incorporated data, and the results obtained are associated with prediction errors and uncertainty. The use of the PBPK modeling technique is still relatively narrow (Khalil & Läer, 2011).

2.4 Silver

Silver has been used as a topical treatment for burns, ulcerations, and infected wounds since the 1800s (Politano et al., 2013). For example, silver sulphadiazine was used as a topical antibacterial agent to treat skin infections in patients suffering from burn injuries

and chronic ulcers. Silver has also been used to treat dental caries as well. In dentistry, silver nitrate was used to prevent caries in primary dentition and permanent molars (Stebbins, 1891), sterilize the cavity and desensitize the dentin (Peng et al., 2012). In 1960, silver fluoride was introduced as an anti-caries agent, presumably for the combined benefits of silver and fluoride. However, due to the black discoloration of caries lesions following the application of silver fluoride compounds, its use has been limited (Rosenblatt et al., 2009). Generally, metal ions' toxic effect on microorganisms contributes to the silver's bactericidal activity. Silver ions themselves are toxic and thus poison the metabolic enzymes and block electron transport systems (Lansdown, 2010).

Penicillin and other antibiotics which emerged in the 1930s were found by health professionals to be superior to silver compounds in their efficacy in treating infections and ease of production, resulting in a significant decline in research and clinical interest in silver. Of late, silver has regained popularity as an antimicrobial agent due to its low toxicity properties, lack of cross-spectrum bacterial resistance, and broad-spectrum (Spadaro et al., 1979).

2.4.1 The Pharmacokinetic of Silver

Silver is a naturally occurring element present widely in human surroundings and combines with other elements such as chloride, nitrate, and sulfide. Silver compounds come in different colors, such as the silver color of pure silver, white color silver nitrate and chloride, black color silver sulfide, and silver oxide (PubChem, 2020).

Silver is present in the air, food, and water. Besides, silver can be found in lozenges and chewing gums to aid in smoking cessation habits (PubChem, 2020). Silver absorption occurs through various routes, like ingestion, inhalation, intra-parenteral insertion of medical devices, and dermal contact. The absorption of silver is low - up to 90 - 99% of

orally ingested silver is not absorbed - and percutaneous absorption of silver ions through intact or damaged skin is also reported to be low. Low concentrations of silver are found in the human body, but it has no trace metal value in the human body. Metallic silver is mainly inert in human body tissues. However, silver and its compounds may dissociate upon contact with the skin surface, body fluids, and secretions. The silver ions are then allowed to be absorbed into the systemic circulation. Biologically active silver ions mainly bind to intracellular proteins as inert complexes and readily bind and precipitate with inorganic cations like chloride and phosphate, which explains the low absorption (Lansdown, 2010). However, it is worth noting that toxic changes occur when the protective mechanisms of metal-binding proteins like metallothionein and their epidermal barrier functions become saturated (Lansdown, 1995).

After a large amount of exposure to silver, the excess accumulates in various organs and tissues, including skin, liver, kidneys, spleen, corneas, gingival, mucous membranes, and nails (Rosenman et al., 1979). An animal study noted that most silver (48%) deposits in the liver (Scott & Hamilton, 1950). Besides that, excess silver accumulates in the blood-brain and blood-CSF barriers without being absorbed into neurological tissue (Lansdown, 2010).

The metabolic pathways of silver are similar irrespective of the route of uptake (Lansdown, 2006). After absorption into the body, silver ions bind to the protein, especially serum albumins and macro-globulins, for the metabolism and distribution to bone and soft tissues. Debate exists on the primary routes of silver metabolism in the human body, its transient or longer-term accumulation in kidney, liver, and bone, and its excretion patterns in bile, urine, hair, and nails (Wan et al., 1991; East et al., 1980).

Silver is excreted mainly through the biliary route, with minimum amounts being exiting through the urinary pathway. Urinary silver measurements may serve as a convenient index of silver absorption in all ways and function as a guide to the total silver

content of the body at blood levels of <100 μg·L−1 (Wan et al., 1991). At higher concentrations, patterns of urinary excretion are irregular. Fecal silver represents that excreted in bile plus the 90% or more ingested with food and not absorbed into circulation (DiVincenzo et al., 1985). The biological half-life for silver is a few days for animals and up to 50 days for the human liver (PubChem, 2020).

2.4.2 Biomarkers of Silver

Biomarkers are widely defined as indicators of signaling events in biological systems or samples. A biomarker of exposure is a xenobiotic substance or its metabolite(s) or the product of an interaction between a xenobiotic agent and some target molecule or cell measured within a compartment of an organism. The preferred biomarkers of exposure are routinely the substance itself or substance-specific metabolites in readily-acquirable body fluid or excrete (National Research Council Subcommittee on & Neurodevelopmental, 1989).

Most people are exposed daily to shallow levels of silver, primarily in food and drinking water and less in the air. The sources of silver are mainly due to its natural occurrence in water and soil (Mintz, 2003). Occupational exposure to silver happens through skin contact and breathing in air containing silver compounds. Besides that, silver in medicines and activities such as jewelry-making, soldering, and photography contribute as minor sources (Lansdown, 2010). Exposure from daily use, such as wearing jewelry or eating with silver-coated flatware, does not generally result in silver being absorbed into the body (ATSDR, 1990).

Silver can be detected in blood, urine, feces, hair, and biopsy specimens using established analytic techniques (ATSDR, 1990; Poitras et al., 2015). The presence of

silver in these samples can be utilized, with differing degrees of accuracy depending on the sample, as a biomarker of exposure to silver compounds.

Feces is the most useful biomarker for silver (ATSDR, 1990) as most of the ingested or inhaled silver exits from the body through it, and only traces are excreted in the urine. Feces as the biomarker, however, presents recruitment challenges due to the very long half-life of silver, analytical difficulties, and computational issues as it would not be able to differentiate between unabsorbed swallowed silver with that excreted in bile following absorption (Lin et al., 2019).

Several studies have evaluated silver levels in blood or urine. For example, (Wan et al., 1991) studied silver levels in the blood and urine samples of adults following applications of silver sulfadiazine cream, a topical cream for treating burn wound sepsis. A recent case report showed increased levels of silver in the blood and urine samples of an adult male who ingested alternative medicines containing silver (Kwon et al., 2009). Heitland and Köster (2006) assessed urinary silver levels in 72 German children to determine background concentrations for biomonitoring purposes. Biomonitoring aims to evaluate exposure to environmental chemicals. Godebo et al. (2019) assessed urinary silver levels from children and adult populations in Africa for the same purpose.

Since 1985, hair analysis has been used to monitor silver exposure (DiVincenzo et al., 1985). Hair analysis is, in fact, potentially helpful for biological monitoring (Bencze, 1990) in assessing the nutritional status of an individual (Lamand et al., 1990) and as an index of environmental exposure (DeAntonio et al., 1982). This is a widely accepted biomarker of chronic exposure to toxic elements (Bermejo-Barrera et al., 1998; Caroli et al., 1994). Blaurock-Busch et al. (2011) evaluated hair silver levels in autistic children and a control group to examine possible risk factors of heavy metals exposure and autistic spectrum disorders. Statistically, the differences between silver levels in both groups were not significant. An earlier study by Adams et al. (2006), which had similar study designs,

also found no significant differences in hair silver levels in both autistic child and control groups.

In comparing blood with hair, trace metals determination in the former has the main advantage of elemental concentrations being about 10-times greater than in serum or urine (Maugh, 1978). Moreover, the use of human hair as a clinical sample presents other benefits such as being readily accessible, easily collected and transported, conveniently stored, handled easily without fear of breakage, and obtained with little or no pain (Al-Delaimy, 2002).

2.4.3 Adverse Effects of Excess Silver

Oral lethal doses (LD_{50}) of 50 and 100 mg/kg of body weight have been observed for different silver salts in mice (Goldberg et al., 1950). In humans, the estimated lethal dose of silver nitrate is at least 10 grams (Furst & Schlauder, 1978). Silver is not carcinogenic in any tissue and should be placed in a "No Risk" category, according to published cytotoxicity tests and *in vivo* experiences (National Toxicology Program, 2011).

Overdose of the silver leads to conditions called argyria and argyrosis. Deposition of silver in the dermis and the silver-induced production of melanin causes argyria. Silver is uniformly deposited in exposed and unexposed areas, with the increased pigmentation becoming more pronounced in areas exposed to sunlight due to the photoactivated reduction of the metal. Although the deposition of silver is permanent, it is not associated with any adverse health effects. No pathologic changes or inflammatory reactions have resulted from silver deposition (Lansdown, 2010). Argyrosis is a condition resulting from the deposition of silver in the eyes. The causative agents are eye drops or make-up containing silver (Greene & Su, 1987). It is worth noting that argyria rarely occurs nowadays and was more common before the discovery of antibiotics.

According to the US Environmental Protection Agency, argyria is likely to be avoided by conserving lifetime exposure of the silver to 1 gram (Agency, 2003). In a PK study of silver diamine fluoride, 2.37 mg was documented as the highest dose of silver applied for three teeth (Vasquez et al., 2012). Dividing 1000 mg by 2.37 mg, the results are more than 400, and this translates into more than 400 applications of such dosage of SDF in a lifetime to produce argyria (Horst et al., 2016). Hence, according to the study, the risk of argyria from SDF application is low (Vasquez et al., 2012). This finding contrasts with (Crystal et al., 2019), who found that an average of approximately 8.08 to 8.71 mg of silver is present in one drop of 38% SDF (Advantage Arrest). Assuming one drop contains around 8 mg of silver, and with a lifetime exposure at 1000 mg, dividing 1000 by eight results in 125 applications of SDF to produce argyria (Horst et al., 2016). The risk of argyria has increased substantially, from 400 to 125 applications. It is crucial to note that the lifetime exposure limit of 1 gram was based on intravenous dosage, whereas the limit based on oral dose is 25 grams. The actual SDF applications to be avoided are more than the calculated 400 and 125 applications, as shown in the above description.

2.4.4 Analysis of Silver

Inductively coupled mass (ICP) is an ionization process that completely breaks-down a sample into its element components and transforms them into ions. In most cases, it is composed of argon gas, and energy is 'coupled' to it using an induction coil to form the plasma (Wilschefski & Baxter, 2019). There are six essential components in an inductively coupled plasma mass spectrometry (ICP-MS) instrument: the sample introduction system; inductively coupled plasma (ICP); interface; ion optics; mass analyzer; and detector (Figure 2.2).

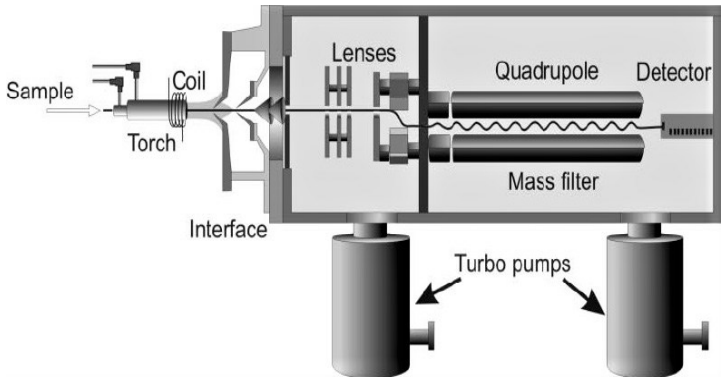

Figure 2.2 Schematic diagram of an inductively coupled plasma mass spectrometry Adopted from Wilschefski and Baxter (2019)

Liquid samples are first nebulized in the sample introduction system creating a fine aerosol that is subsequently transferred to the argon plasma. The high-temperature plasma atomizes and ionizes the sample, generating ions extracted through the interface region and into a set of electrostatic lenses called ion optics. The ion optics focus and conduct the ion beam towards the quadrupole mass analyzer, which then sets apart ions based on their mass-charge ratio (m/z). These ions are measured at the detector (Wilschefski & Baxter, 2019). ICP-MS instruments are primarily designed to analyze liquids. Solid samples can be analyzed directly using electrothermal vaporization or laser-ablation (Wilschefski & Baxter, 2019).

ICP-MS is a highly sensitive analytical technique for measuring trace elements of clinical interest in biological samples. It has high sensitivity, comprehensive linear dynamic range, wide elemental coverage, multi-element capability, high sample throughput, and simple sample preparation. These features are critical in clinical and experimental research (Wilschefski & Baxter, 2019).

In the literature, three studies reported normal reference values using ICP-MS for silver levels in urine (Godebo et al., 2019; Heitland & Köster, 2006; Lin et al., 2019). Table 2.4 summarizes the details of the study.

Table 2.4 Normal ranges of urinary silver levels reported in the literature

Author and year	Normal Range (µg/L)	Analytical Method	Subjects	Country
Heitland & Köster (2006)	<0.008-0.05	ICP-MS	72 children (occupationally unexposed)	Germany
Lin et al. (2019)	<0.05-0.06	ICP-MS	16 adults exposed to SDF	USA
Godebo et al. (2019)	<0.01-0.2	ICP-MS	386 adults and children (exposed to naturally occurring toxic and essential elements)	Ethiopia

For silver levels in hair samples, studies by (Adams et al., 2006; Blaurock-Busch et al., 2011) reported normal ranges in children in the United States and the Kingdom of Saudi Arabia, respectively (Table 2.5). Another study from France evaluated silver levels in hair samples but did not specify whether they were collected from adults or children (Goullé et al., 2005).

To the best of the author's knowledge, no studies have been reported on the levels of silver in hair and urine samples in the Malaysian population.

Table 2.5 Normal values of hair silver levels reported in the literature

Author and year	Hair silver level (µg/g)	Analytical Method	Subjects	Country
Blaurock-Busch (2011)	Mean: 0.39	ICP-MS	25 children (age:6.25±2.31)	Kingdom of Saudi Arabia

Author and year	Hair silver level (µg/g)	Analytical Method	Subjects	Country
Adams et al. (2006)	Mean: 2.13	ICP-MS	40 children (age:7.5±3.0)	United States
Goulle et al. (2005)	Range: 0.02 – 1.31	ICP-MS	45 healthy volunteers	France

Table 2.5 Continued

2.5 Fluoride

A fluoride is a form of a chemical element, fluorine. The primary source of fluoride intake in most parts of the world is food. Water also contains fluoride (World Health Organization, 2019b). While almost all kinds of food have trace quantities of fluoride, fish products containing bones, such as canned salmon and sardines, can present high fluoride levels. Levels in meat, fruit, and vegetables are usually low. However, tea plants may absorb fluoride from the soil in substantial quantities; therefore, tea consumption can lead to high fluoride intake (World Health Organization, 2017). Currently, the fluoride present in drinking water has different concentrations throughout the world. The optimal drinking-water concentration is generally within the range of 0.5-1.0 mg/L (O'Mullane et al., 2016; World Health Organization, 2017). In Malaysia, fluoride concentrations in water ranged from 0.5 to 0.7 ppm (Ministry of Health Malaysia, 2006).

Fluoride is found in natural sources and industrial processes. Commonly, it is distributed in the earth's crust, mainly as the minerals fluorspar, cryolite, apatite, mica, hornblende, and fluorite (O'Mullane et al., 2016). Fluoride is present in dust from fluoride-containing soils, gaseous industrial waste, and pesticides containing fluoride. It can be distributed widely and transported by wind over long distances before being deposited. Therefore, fluoride concentrations differ by geographical region (O'Mullane et al., 2016). Industrial sources contribute to airborne fluoride in urbanized areas. Primary

sources include the manufacture and use of phosphate fertilizers, phosphate ores, aluminium, steel, and other metal production facilities (World Health Organization, 2019b).

Fluoride is widely used for checking dental decay in several countries. It is the only compound recognized by the US FDA to prevent dental caries, though not all fluoride-containing products are recognized for protection against caries (Carey, 2014). Across the world, fluoride-containing products like toothpaste, mouth rinses, topical fluoride varnish, and gel are effective methods to control dental caries. WHO recommends fluoridated toothpaste containing between 1000 and 1500 ppm fluoride as the standard measure to prevent dental caries (O'Mullane et al., 2016).

It was well acknowledged in the 1980s that fluoride controls caries primarily through its topical effect. During an acid challenge, the fluoride present in low, continual concentrations (sub-ppm range) in oral fluids were found to absorb into the surface of the apatite crystals, preventing demineralization. When the pH is re-established, fluoride traces in the solution will make it highly supersaturated in fluorhydroxyapatite, speeding up remineralization. The mineral formed under its partially dissolved nucleating action will then preferentially include fluoride and exclude carbonate, making the enamel more resistant to future acidic attacks (Featherstone, 1999). Besides, topical fluoride can also impart an antimicrobial effect. Fluoride concentrations, as discovered in dental plaque, have biological activity on critical virulence factors of *S. mutans in vitro*, such as acid production and glucan synthesis. Still, the *in vivo* implications of this remain unclear. Evidence also strengthens fluoride's systemic mechanism of caries prevention in pit and fissure surfaces of first permanent molars when integrated into these teeth pre-eruptively (Buzalaf et al., 2011).

2.5.1 The Pharmacokinetic of Fluoride

Generally, fluoride enters the body through the gastrointestinal tract and is absorbed quickly in the stomach without the presence of specialized enzymes (Whitford, 1996). It crosses epithelium as undissociated hydrogen fluoride. Most fluoride absorption occurs in the small intestine in addition to crossing the stomach as undissociated acid (Villa et al., 2010; Villa et al., 2009; Villa et al., 1993).

The proportion of fluoride absorption from the stomach is directly associated with its contents' acidity (Whitford & Pashley, 1984). However, several other determinants affect the absorption rate, including the solubility of the ingested fluoride compound. The more soluble the compounds, the faster the absorption rate, such as sodium fluoride (NaF) and hydrogen fluoride. Calcium fluoride (CaF2) and magnesium fluoride (MgF2), being less soluble, would slow down absorption. (Ekstrand & Ehrnebo, 1983). Soon after the fluoride is absorbed, plasma fluoride levels increase (at 10 minutes), reaching peak levels at 60 minutes (Whitford, 1996). A return to basal levels is reached within 11 to 15 hours (Ekstrand & Ehrnebo, 1983).

Fluoride is swiftly deposited in the skeleton or excreted via the kidneys once it reaches plasma (Dunipace et al., 1995). Fluoride skeletal uptake is also influenced by bone modeling, remodeling, and age (Dunipace et al., 1995). The extent of fluoride retained in the skeleton is inversely proportional to the age of the individual. In a person without previous exposure to fluoride, the amount of fluoride absorbed increases until saturation is attained (Hodge et al., 1970). In the skeleton, fluoride can be accumulated in the adsorbed layers, the crystal structures, or the bone matrix. Once fluoride is incorporated, and when bone saturation is approached, the fluoride can be slowly removed (Likins et al., 1956). Salivary levels of fluoride increase together with plasma levels as fluoride is secreted in saliva. Salivary fluoride level is essential in preventing dental caries even

though it only ranges between 0.01 to 0.06 ppm in the body when fluoride exposure takes place (Ingram et al., 2005).

The fluoride is excreted mainly via the kidneys, with a minimal amount excreted through feces when it is not stored in bone. Both urinary flow and pH are involved in controlling the renal clearance of fluoride from plasma (Whitford, 1996). The proportion of ingested fluoride excreted in the urine (fractional urinary fluoride excretion) is also regulated by age and demonstrates clear and distinct nocturnal and diurnal patterns (Villa et al., 2010; Villa et al., 2009). For healthy adults, about 50% of absorbed fluoride is retained by uptake in hard tissues, and the remaining 50% is eliminated in urine. About 80% of fluoride is retained in young children due to increased uptake by the developing skeleton and teeth (Ekstrand, Fomon, et al., 1994; Ekstrand, Ziegler, et al., 1994). Figure 2.3 illustrates the fluoride metabolism flowchart.

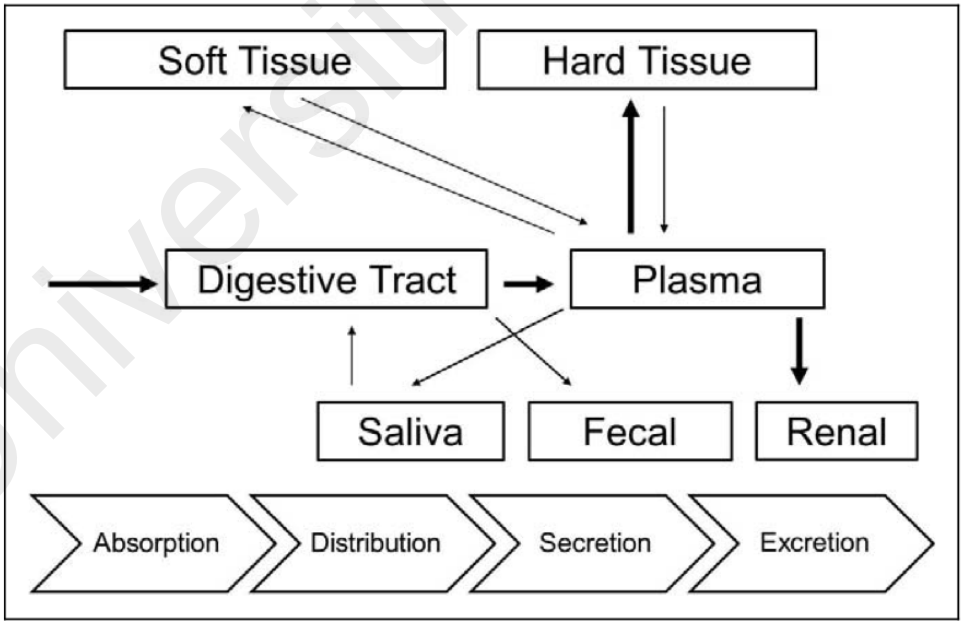

Figure 2.3 Fluoride metabolism flowchart
Adopted from Martinez-Mier et al. (2012)

2.5.2 Biomarkers of Fluoride

Contemporary biomarkers of fluoride are blood, bone surface, saliva, milk, sweat, and urine. Nails and hair are considered the biomarkers of recent exposure, while bone and teeth represent biomarkers of historical exposure. According to WHO, urine is the most helpful biomarker of contemporary fluoride exposure (World Health Organization, 2014).

Daily urinary fluoride excretion (DUFE) is a valuable biomarker of contemporary fluoride exposure as known quantities of ingested fluoride are excreted in urine (Villa et al., 2010). The range of optimal exposure to fluoride/total daily fluoride intake (TDFI) is 0.05 - 0.07 mg/kg body weight/day. Low TDFI is 0.02 mg/kg body weight whereas high TDFI is 0.1 mg/kg body weight. DUFE is calculated based on TDFI using the formula DUFE = TDFI x 0.35 + 0.03 (Rugg-Gunn et al., 2011; World Health Organization, 2014).

Fluoride concentrations found in parotid and submandibular saliva but not in the whole saliva have also been suggested as being associated with plasma fluoride concentrations. To date, insufficient data is available to demonstrate a normal reference value of fluoride levels in ductal saliva. Hence, salivary fluoride level is not an appropriate contemporary biomarker of fluoride exposure based on current evidence (Rugg-Gunn et al., 2011).

2.5.3 Adverse Effects of Excess Fluoride

A high level of fluoride intake can provoke toxic effects by binding with calcium and disrupting proteolytic and glycolytic enzyme activity. Hydrofluoric acid is generated in the stomach through the reaction of ingested fluoride with gastric acid. Therefore, immediate side effects such as abdominal pain, excessive saliva, nausea, and vomiting may occur after acute exposure to excessive fluoride. Muscle spasms and epilepsy may also happen, while death due to respiratory paralysis is possible as well (World Health Organization, 2019b).

The primary effect of long-term ingestion or inhalation of high fluoride concentrations is fluorosis (World Health Organization, 2019b). While a total daily fluoride intake (TDFI) of between 0.05 - 0.07 mg/ kg body weight is the optimum range for dental health, the risk for dental fluorosis increases when the TDFI exceeds 0.1 mg/kg body (European Food Safety, 2005). Fluorosis can occur in the enamel and skeleton. Progressive accumulation of fluoride over the years results in skeletal fluorosis, with stiffness and pain in the joints being among the early symptoms. Over the long term, crippling skeletal fluorosis characterized by osteosclerosis, calcification of tendons and ligaments, and bone deformities may result. Evidence from India and China shows the risk of skeletal fluorosis and bone fractures increasing excessively at a total fluoride intake of 14 mg/day. It also suggested an increased risk of skeleton effects at a total intake above 6 mg/day (World Health Organization, 2017).

Enamel fluorosis relates to fluoride-related changes in enamel, which occur during enamel development. These changes become more severe with increasing fluoride intakes and exposure time. The severity of fluorosis is related to the fluoride concentration in the plasma, considered to be in equilibrium with the tissue fluid that bathes the enamel organ (Angmar-Månsson et al., 1976). Plasma fluoride levels are influenced by many components, including total fluoride intake, type of intake (i.e., ingested vs. inhaled), renal function, rate of bone metabolism, metabolic activity, etc. (Angmar-Månsson et al., 1990). Besides, genetic factors appear to influence the severity of enamel fluorosis in mice. As enamel fluorosis is due to the high intake of fluoride during tooth formation, it can only occur in children. For the whole permanent dentition (excluding the third molars), the window of vulnerability for fluorosis development is the first 6 to 8 years of life (Bhagavatula et al., 2016). The appearance of white, mottling areas in the enamel is characteristic of enamel fluorosis. Aesthetically it is a potential problem while in more severe forms, decreased mineralization of the enamel results in stained and pitted teeth.

To date, four major risk factors for dental fluorosis have been identified, i.e., fluoridated drinking water, fluoride supplements, fluoride toothpaste, and infant formula (Buzalaf, 2018).

Currently, there is no evidence that fluoride is carcinogenic. Fluoride levels in drinking water are not associated with an increased risk of bone cancers in humans (O'Mullane et al., 2016). However, studies on rats show an increased risk of osteosarcoma following very high fluoride exposure levels ("NTP Toxicology and Carcinogenesis Studies of Sodium Fluoride," 1990). Based on epidemiological studies of pregnancy outcomes, there is no relationship between the occurrence of Down's syndrome or congenital malformation with fluoridated drinking-water consumption (WHO, 2017).

2.5.4 Analysis of Fluoride

Fluoride analysis in fluid samples is performed using an ion-selective electrode (ISE) (Figure 2.4). An ISE is an electroanalytical sensor of the activity of a specific ion in a solution. This activity is converted into an electrical potential, which can be measured by a voltmeter. The sensing part of the electrode is usually made as an ion-specific membrane, along with a reference electrode.

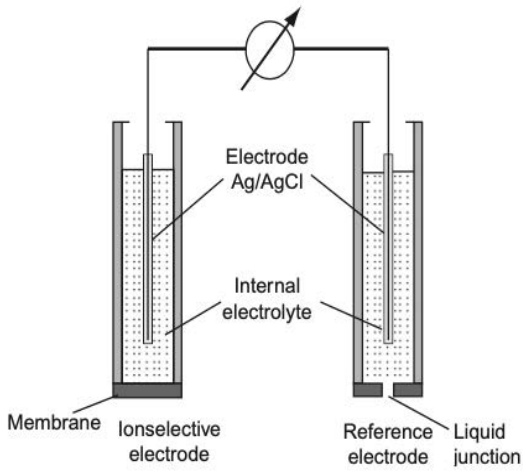

Figure 2.4 Schematic diagram of an ion-selective electrode (ISE) measurement Adopted from Wojciech Wroblewski at CSRG, University of Warsaw, Poland

The principle of ISE is the generation of the difference in its own and the reference electrode's electrical potential. The resulting output potential corresponds to the concentration of specific ions in the solution. The concentration is a measure of the number of ions in a specific volume. It is worth noting that the electrode is assumed to measure the activity of the ions rather than their concentration. The ionic activity and concentration are practically identical in a dilute solution whereas activity and concentration may differ in solutions containing many ions. Hence dilute samples are favored for measurement with ISEs. The solution can be 'fixed' to ensure the equality of the activity and concentration of the ions. The addition of an Ionic Strength Adjustment Buffer (ISAB), a constant concentration of an inert electrolyte, to the solutions being tested aids in this purpose. The direct measurement of the concentration of ions results (Toledo, 2018).

The ISE is typically applied in agriculture, biomedical and clinical labs, beverages, chemistry, detergents, education, electroplating, environment, explosives, food, mining, paper and pulp, pharmacology, power generation and water (Toledo, 2018).

Generally, fluoride measurements are performed potentiometrically, using a fluoride electrode in conjunction with a standard single-junction, sleeve-type reference electrode, and a pH meter with an expanded millivolt scale or a selective ion meter that has a direct fluoride concentration scale. Fluoride ion activity depends on the solution's total ionic strength, pH, and fluoride complex species. Adding an appropriate buffer provides an almost uniform ionic strength background, adjusts the pH, and breaks up complexes so that the electrode measures concentration. An example is the total ionic strength adjusting buffer (TISAB). A reliable pH/ISE meter is compatible with specific fluoride ion electrodes and in determining the urine pH required for sample analysis (Toledo, 2018).

An analysis method should be validated, when necessary, to demonstrate that its performance characteristics are adequate for a particular purpose. There is a critical need to validate analytical methodologies in certain conditions, such as those methods that are not standard, designed, or developed by a laboratory, as well as standard but used outside their intended scope or modified (B. Magnusson, 2014).

The two main approaches to methods validation are the inter-laboratory comparison and the single-laboratory approach. In the case of a method being developed that will have wide-ranging use, perhaps as a published standardized procedure, a collaborative study involving a group of laboratories is probably the preferred way of carrying out the validation. However, if the method is developed for use in only one laboratory due to the lack of general interest in it or because other laboratories are competitors, the single-laboratory approach would be appropriate (Thompson et al., 2002).

2.6 Method Validation

An analysis method should be validated, when necessary, to demonstrate that its performance characteristics are adequate for a particular purpose. There is a critical need to validate analytical methodologies in certain conditions, such as methods that are not standard, designed, or developed by a laboratory; standard but used outside their intended scope or modified (B. Magnusson, 2014).

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2.6.1 Tools for Methods Validation

Validation tools include blanks, spike materials/solutions, and measurement standards. The use of blanks enables assessing how much of the measured signal is attributable to the analyte and how much to other causes. Various types of blanks are available. Reagent blanks are used during the analytical process (including solvents used for extraction or dissolution). The analysis of reagent blanks is necessary to determine whether they contribute to the measurement signal. Sample blanks are essentially sample matrices with no analyte present, e.g., a human urine sample without a specific drug of abuse. Sample blanks may be challenging to obtain but are necessary for providing a practical gauge of interferences faced in the analysis of test samples (B. Magnusson, 2014).

Routine test samples are materials or solutions in which the analyte(s) of interest have been purposefully added. The routine test sample may have the analyte of interest to start with. Hence, the analyst needs to be cautious to ensure the spiking does not cause the analyte levels to exceed the method's working range. Spiking of the routine test sample enables quantifying the heightened response to the analyte (B. Magnusson, 2014).

Measurement standards can be anything in which a particular parameter or property has been characterized to the extent it functions as a metrological reference. Examples of measurement standards include reference materials (RM) and certified reference materials (CRM). RMs could be any material used as a basis for reference and could consist of laboratory reagents of known purity, industrial chemicals, or other artifacts. CRMs have a more strictly controlled characterization of the parameter of interest than for an RM. Besides, the characterized value is certified with documented metrological traceability and uncertainty (International Organization for Standardization, 2015).

2.6.2 The Process of Methods Validation

Firstly, the laboratory needs to identify a suitable existing method or develop/modify a method if necessary. Secondly, the laboratory will then identify and evaluate relevant performance characteristics and check them against the analytical requirement. Finally, the validation process ends with a conclusion and statement of whether the analytical requirement is met. If the analytical requirement is not met, further method development is necessary. This development and evaluation process continues until the method is deemed capable of meeting the prerequisite (B. Magnusson, 2014). Figure 2.5 shows the method validation process.

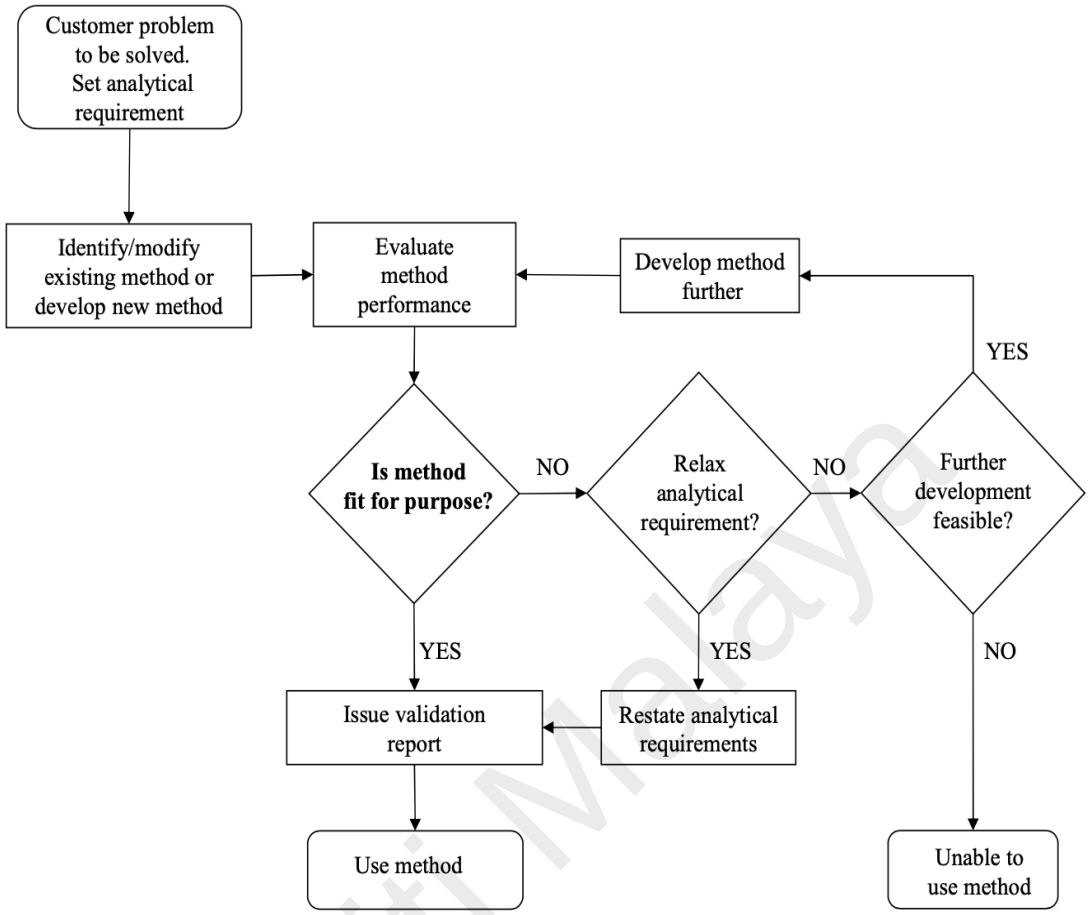


Figure 2.5 The methods validation process
Adopted from Magnusson (2014)

2.6.3 Methods Performance Characteristics

Methods performance characteristics include the limits of methods detection, quantification, recovery, and precision (B. Magnusson, 2014). Table 2.6 summarizes the various definitions of methods' performance characteristics.

Table 2.6 Methods performance characteristics

Methods Performance Characteristics	Definition
Method detection limit (LOD)	Minimum measured concentration of a substance reported with 99% confidence that the measured concentration is distinguishable from method blank results.
Limit of quantification (LOQ)	Smallest analyte concentration which can be quantitatively analyzed with reasonable reliability by a given procedure.
Recovery	Process efficiency of an analytical process, reported as a percentage (%) of the known amount of analyte carried through the sample extraction and processing steps of the method.
Precision	Closeness of agreement between measured quantity values obtained by replicate measurements on the same analytes under specified conditions. It is a measurement of the degree of agreement among replicate analyses of a sample, usually expressed as a standard deviation (SD) Measurement repeatability' and 'measurement reproducibility' represent the two extreme measures of precision which can be obtained. Repeatability, expected to give the smallest variation in results, is a measure of the variability in results when a measurement is performed by a single analyst using the same equipment over a short timescale. Reproducibility, expected to give the largest variation in results, is a measure of the variability in in results between laboratories.
Linearity	Linearity is the ability of the method to elicit results that are directly proportional to analyte concentration within a given range.

In method validation, the focus is on LOD and LOQ. To obtain LOD, it must be based on the analysis of samples that have been taken through the whole measurement procedure using results calculated with the same equation as for the test samples. Replicate measurements of reagent blanks spiked with low concentrations of analyte are an acceptable method. It is essential that this standard deviation represents of the precision obtained for typical test samples and that a sufficient number of replicate measurements is made (10 is usually recommended) to give a reliable estimate. LOD is calculated as LOD = 3 × standard deviation from the replicates of sample-spiked urine. LOQ is calculated by most conventions to be the analyte concentration corresponding to the obtained standard deviation at low levels multiplied by a factor k_Q. The International Union of Pure and Applied Chemistry (IUPAC) default value for k_Q is ten, and LOQ = k_Q x standard deviation^A. Standard deviation^A (SD^A) is from the replicates of sample-spiked urine from LOD.

In United Kingdom (UK), a study evaluated the urinary level of 61 elements, including silver, in 132 adults without occupational exposure and found the reported LOQ for urinary silver levels at 0.029 µg/L (Morton et al., 2014). In Germany, another study by Heitland and Köster (2006) reported the LOQ for urinary silver at 0.008 µg/L. Table 2.7 shows the results of these studies.

Table 2.7 LOQ for urinary silver levels reported in the literature

Author and year	LOQ (µg/L) silver level in urine	Analytical method	Subjects	Country
Morton et al. (2014)	0.029	ICP-MS	132 adults	UK
Heitland et al. (2006)	0.008	ICP-MS	72 children, 87 adults	Germany

Following SDF application, (Lin et al., 2019) reported that the LOD for urinary fluoride was 0.01 mg/L, while for urinary silver levels, the LOD was 0.05 µg/L (Lin et al., 2019). According to the NIOSH Manual of Analytical Methods, the LOD of urinary fluoride was 0.01 mg/L as well (CDC, 1994).

Critical requirements for quantitative analysis are the accuracy and the precision (also known as repeatability and reproducibility) obtained from actual lab data, which are reflected in the measurement uncertainty. Measurement 'accuracy' expresses the closeness of a single result to a reference value. Method validation seeks to investigate the accuracy of results by assessing both systematic and random effects on single results. Accuracy is, therefore, generally studied as two components: 'trueness' (recovery) and 'precision' (B. Magnusson, 2014). Accuracy should be assessed using a minimum of 9 determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration). Prepare 3 sample preparations of a composited sample containing a known quantity of added analyte ("matrix spike") so that the expected concentrations range at least 80% - 120% of expected content (Food and Drug Administration, 2020).

Precision is the agreement between a set of replicate measurements without the assumption of knowledge of the actual value. Repeatability expresses the precision under the same operating conditions over a short period. The precision is described by statistical methods such as a standard deviation or confidence limit of test results. Repeatability should be assessed using a minimum of nine determinations covering the specified procedure range or a minimum of six determinations at 100% of the test concentration unless otherwise specified. Intermediate precision expresses within-laboratory variations, such as different days, different analysts, and different equipment (Food and Drug Administration, 2020; Shabir, 2004). Intermediate precision gives an estimate of the variation in results when measurements are made in a single laboratory but under

conditions that are more variable than repeatability (Food and Drug Administration, 2020). Reproducibility expresses the precision between laboratories (Food and Drug Administration, 2020). Reproducibility is precision between measurement results obtained at different laboratories. It is not always needed for single-lab validation but important if the method is used in several laboratories. Sometimes reproducibility is referred to as within laboratory reproducibility at the level of intermediate precision (Belouafa et al., 2017).

Linearity is the method's ability to elicit results directly proportional to analyte concentration within a given range. It is measured by preparing and analysing a set with a minimum of 5 concentrations of analyte standard across a minimum range based on the type of analysis (Food and Drug Administration, 2020). For example, the range for assay of a drug substance is from 80% to 120% of the test concentration. The determination is performed, and a standard curve is generated. The correlation coefficient, y-intercept, slope of the regression line should be reported. The linear regression coefficient of determination r^2 greater than or equal to 0.995 is the acceptance criteria (Food and Drug Administration, 2020).

CHAPTER 3: MATERIALS AND METHOD

3.1 Overview of Research Design

The current study is a clinical and laboratory study aimed at assessing the pharmacokinetic characteristics of silver diamine fluoride applied on the surface of carious teeth. Silver exposure of the children was evaluated by examining silver levels in hair and urine samples. In contrast, fluoride exposure was assessed based on fluoride levels in urine samples only.

3.2 Ethical Approval

Prior to commencing the research project, regulatory permissions were obtained. This research project was sponsored by the University of Malaya, Dental Postgraduate Research Grant (DPRG) with grant project number DPRG 18/19. Ethical approval for the research was received from the Faculty of Dentistry Medical Ethics Committee (FDMEC), with FDMEC reference of DF CD1914/0065/20157 (P) (Appendix V).

3.3 Anonymity and confidentiality

Data collected for the study was treated as confidential. The identity of participants was anonymized so that no names were associated with their various forms of data. Participants' clinical records and biological samples retrieved for the study were coded to ensure anonymity. The decoding sheets were held separately from the data itself. The content of individual details, clinical records, and results was not disclosed to any third party. Both urine and hair samples were discarded as biological waste within a month.

3.4 Sampling Method

Patients who attended the Paediatric Dental Specialist Clinic, Faculty of Dentistry at the University of Malaya, were screened for eligibility to enroll in the study. The convenience sampling method was used. The study was conducted in this clinic from July 2020 to July 2021. The recruitment of subjects was based on the following inclusion and exclusion criteria:

3.4.1 Inclusion Criteria

1) Patient factors:
    a) Children between 4 to 10 years of age
    b) Medically fit and healthy
    c) Had obtained written consent and permission to participate in the study
    d) Presented with a score of 1 or 2 in the Frankl's behavior rating scale, in which conventional restorative treatment is not indicated
2) Tooth factors:
    a) Present with at least one decayed tooth, with no signs and symptoms of pulpitis
    b) Radiographically, the caries lesion presented as radiolucency involving dentin, without pulpal involvement
    c) Having cavitated caries lesions on any surface, as long as they were accessible with a brush for SDF application. Orthodontic separators may be used to help gain access to proximal lesions.

3.4.2 Exclusion Criteria

1. Children taking prescribed medications/over the counter medications/supplements
2. Children allergic to silver or fluoride
3. Children having oral ulcer/mucositis that have not healed completely

4. Children whose teeth had been restored with amalgam or stainless-steel crowns
5. Children whose teeth had been recently restored with glass ionomer cement

3.5 Sample size calculation

A statistical power analysis was performed for sample size estimation based on Lin et al. (2019) (N=16) to analyse silver and fluoride levels in urine and blood after silver diamine fluoride treatment. This study used a similar methodology and grouping concurrent with the objectives of this study. With an $\alpha = 0.05$ and power=0.95, Wilcoxon signed-rank test (due to the non-normally distributed data in Lin et al. (2019)) was used to calculate the sample size. The projected sample size (N) was 13 (Appendix A). After considering the 20% drop-off rate, the proposed sample size of 15 was deemed adequate for this study's objectives.

3.6 Clinical Examination

Three investigators performed screening for study participants by routine dental examination at a dental clinic (LSP, SB, NHZA). All investigators had undergone the International Caries Detection and Assessment System (ICDAS) training and calibration workshop held at the Faculty of Dentistry, University of Malaya. SB was the gold standard in dental charting.

After the screening, inter-rater reliability was tested using Fleiss multirater kappa to determine agreement among all investigators (Appendix B). These investigators were responsible for the review of SDF-treated teeth as well. A dental radiograph was taken following the best clinical practice guidelines for prescribing dental radiographs by EAPD (Kühnisch et al., 2020).

Materials

Silver Diamine Fluoride (SDF), Riva Star, SDI Ltd, Australia, 56,900 ppm fluoride

Simple dental armamentarium involved were:

- Dental mirror
- Periodontal probe (WHO CPITN probe/William probe)
- Slow-speed handpiece, and
- Rubber cup with prophylaxis paste.

Teeth were cleaned with prophylaxis. Dental charting was performed using ICDAS (Nigel B. Pitts, 2014). When charting the carious teeth, each investigator could select from only one of the eight categories, as follows:

1. Sound tooth surface
2. First visual change in enamel
3. Distinct visual change in enamel
4. Localized enamel breakdown with no visible dentin
5. Underlying dark shadow from dentin
6. Distinct cavity with visible dentin
7. Extensive distinct cavity with visible dentin
8. Presence of restorations

The patients and parents/guardians were informed about the study's rationale and aims, procedures, possible benefits and risks or complications, anonymity, and the voluntary basis. Possible benefits included free SDF treatment as well as simple restorations and topical fluoride application. A toothbrush and toothpaste set were given to each participant as a token of appreciation. Potential risks included temporary ulcers from the direct contact of SDF with the gums. Cotton or gauze was placed to protect the

gums during treatment. Upon application, a child may notice a metallic taste, which will go away rapidly. The patients and parents/guardians were allowed to ask questions related to this study. A participant information sheet was given (Appendices P and Q), and informed consent (Appendices R and S) was obtained. The participant information sheet and informed consent were available in both English and Malay language.

3.6.1 Study Procedure

The SDF application procedure was performed per the American Association of Paediatric Dentistry ("Chairside Guide: Silver Diamine Fluoride in the Management of Dental Caries Lesions," 2018). The study procedure was summarized and shown in Figure 3.1.

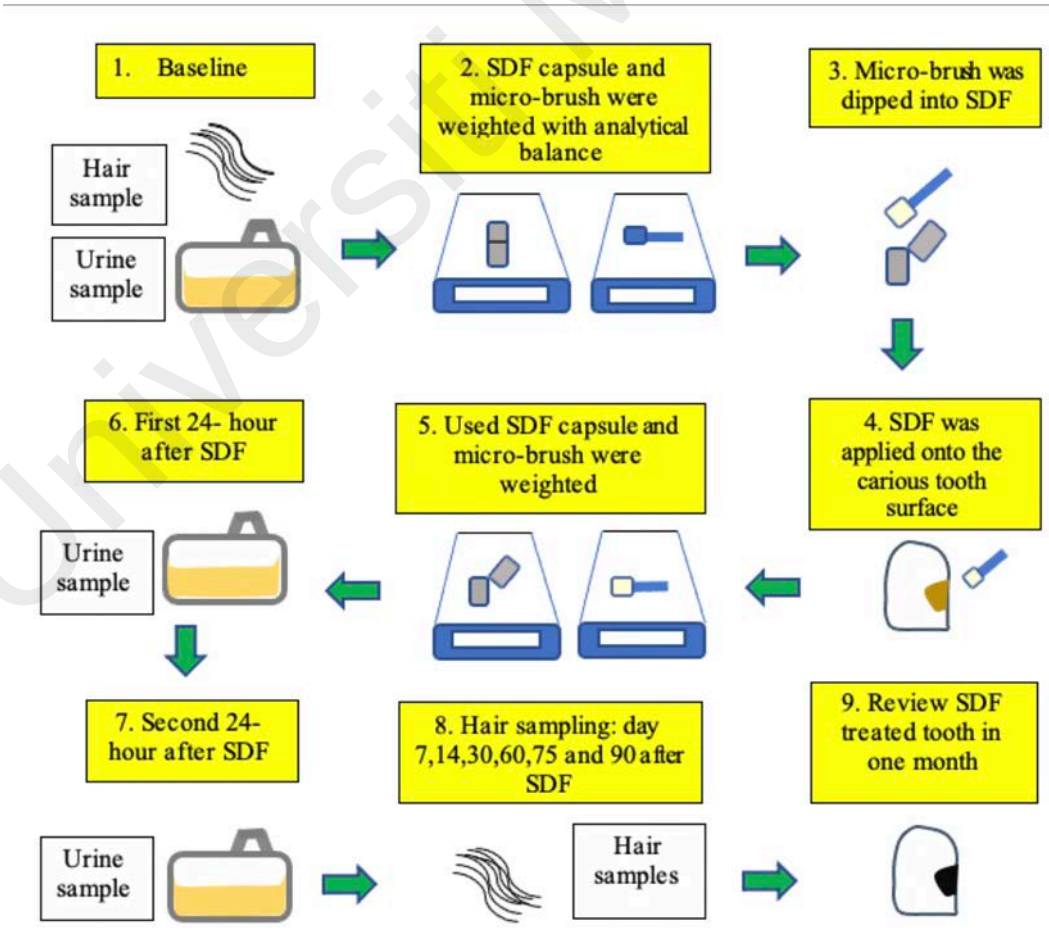


Figure 3.1 Flow diagram for the study procedure

Study procedure:

1. The SDF capsule and a micro-brush were weighted with an analytical balance machine (Figure 3.2).

2. Gross food debris was removed from teeth cavities to allow better SDF contact with denatured dentin.

3. A protective coating of vaseline was applied to the lips and skin to prevent a temporary henna-appearing tattoo that can occur if soft tissues come into contact with SDF.

4. The areas to be treated were isolated with cotton rolls or other isolation methods.

5. No more than one capsule of SDF was used for the entire appointment.

6. The lesion was dried with gentle flows of compressed air.

7. The micro brush was bent and dipped into the SDF capsule. The careful application was performed with it to prevent intraoral and extraoral soft tissue exposure.

8. SDF was applied directly to only the affected tooth surface.

9. Application times were limited to at least one minute if possible. When they were shorter in very young children who were more challenging to manage, the patients were monitored carefully at post-operative and recall visits to evaluate arrest and consider reapplication.

10. Gentle flow of compressed air was applied until the medicament dried.

11. The tooth was then kept isolated for as long as three minutes. A picture of teeth treated with SDF is shown in Figure 3.3.

12. The used SDF capsule and micro brush were weighted with the analytical balance machine (Figure 3.2).

13. Follow-up was performed four weeks after the initial treatment to check the arrest of the lesions treated. Reapplication of SDF was indicated during the review if the treated lesions did not appear arrested (dark and hard).

14. Caries lesions were restored after treatment with SDF if the child was co-operative. Biannual reapplication was done when lesions were not restored after SDF therapy.

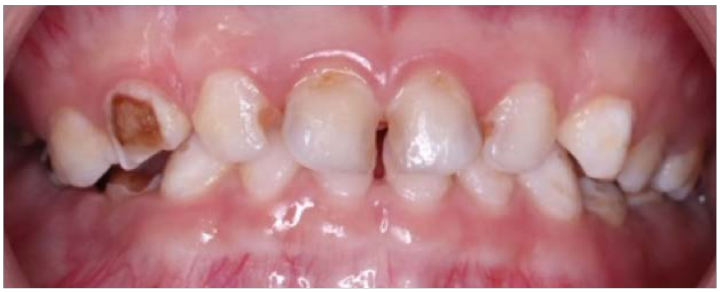
Weight of SDF capsule before treatment

Weight of SDF capsule after treatment

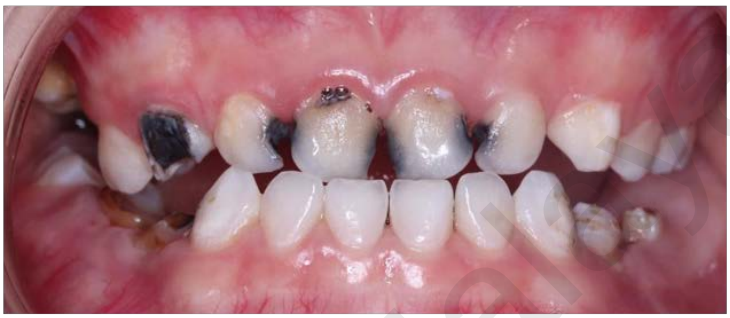
Weight of micro-brush before treatment

Weight of micro-brush after treatment

Figure 3.2 Weighting processes for the SDF capsule and micro-brush using the Analytical Balance



Front view of anterior teeth (51,52,53,61,62) prior to SDF treatment



Front view of anterior teeth after the SDF treatment

Figure 3.3 Clinical pictures of teeth treated with SDF

3.7 Sample Collection

Sample collection in this study comprised of urine and hair samples. A day before SDF treatment the patients' teeth were brushed with fluoride-free toothpaste and continued for two days. The patient and their parents were advised to avoid high fluoride content food a day before SDF treatment and continued for two days after SDF treatment, until the urine collection had ended.

3.7.1 Urine Sample Collection

The urine samples were collected a total of three times. Three units of urine containers and iceboxes were provided. An example of the urine samples and containers is shown in Figure 3.4.

Figure 3.4 Urine container with a 24-hour urine sample

Each of the urine samples was collected over 24 hours. The baseline 24-hour urine sample was collected before SDF treatment, followed by the first and second 24-hour urine samples after treatment. The method for collecting the 24-hour urine was as follows:

Collection procedure for the 24-hour urine samples:

1. On the day of the 24-hour urine sample collection, the children emptied their bladders (urinate) into the toilet immediately after waking up.
2. The parents recorded the date and start time. Urine collection began with the empty bladder.
3. From this time on (the first 24-hour urine collection), urination was done with a plastic collection container. The urine was then poured into the 24-hour urine container.
4. The urine was stored in the 24-hour urine container in the cooler box with ice. The urine was constantly kept cold. The lid on the urine container was tightly sealed.
5. The urine was collected and poured into the 24-hour urine container each time the child urinated during the day and night.
6. The child was asked to urinate when waking up the following morning (close to the same time as the first day). This first urine specimen was collected and added to the 24-hour urine container. This marked the end of the first 24-hour collection. No other urine was added to the container.

7. The date and time of the last urine specimen were noted on the 24-hour urine container together with the child's coded identity.

8. Collection of the first 24-hour urine samples after the SDF treatment:

a. The same procedures as above were repeated except for the starting time, which was now immediately after SDF treatment.

b. The end time was the last urination time corresponded with the starting time of the present urine collection.

9. Collection of the second 24-hour urine samples after the SDF treatment:

1. The same procedure was repeated except for the starting time, which was now immediately after the ending time of the first urine sample collection after SDF treatment.

2. The ending time was the last urination time corresponding with the starting time of the present urine collection.

10. The principal investigator (LSP) visited the subjects' house to take the urine samples once they were ready and sent them (kept cold in ice-boxes) to the laboratory.

3.7.2 Hair Sample Collection

Hair samples were collected before SDF treatment and 7, 14, 30, 60, 75, and 90 days after treatment. The hair at the posterior vertex part of the head was kept at least 5cm from the scalp for three months. If haircuts were required, the hair in this area was not trimmed. Hair sample collection was performed by the principal investigator (LSP). Figure 3.5 showed an example of hair samples.

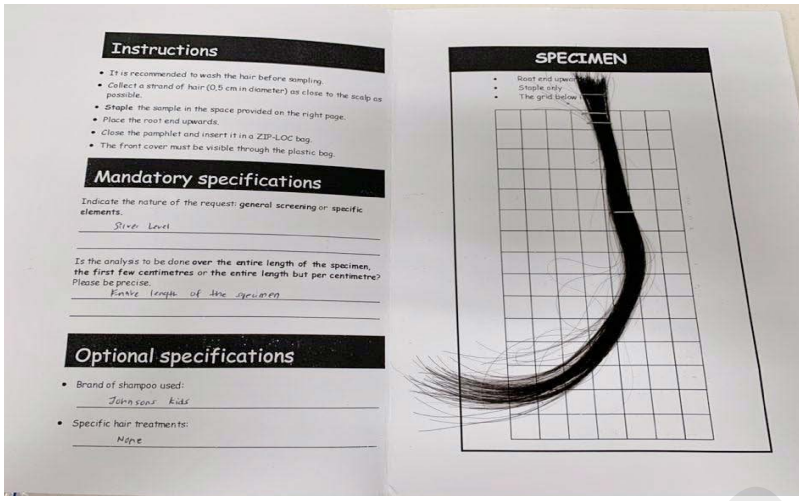


Figure 3.5 Hair samples in the hair sampling card

Hair sample collection method (Cooper et al., 2012):

1. Identity details of patients were confirmed.

2. The sample was cut from the posterior vertex region of the head, as close as possible to the scalp, since this region has the least variation in growth rates.

3. In general, head hair is estimated to grow approximately 1.0 cm per month.

4. A lock of hair of 0.5cm thickness was taken (based on the Centre de toxicologie du Québec (CTQ) laboratory's instructions).

5. The hair sample was then placed in a hair sampling card provided by the laboratory (Appendices T, U), with the roots facing the top and the hair ends facing the bottom edge of the card.

6. The hair sample was secured on the card with staplers. The patient's code and date of hair sampling were written clearly on the card.

7. The card was then placed into a zip lock bag.

8. The patient's identification code and date of hair sampling were written again on the zip lock bag.

3.7.3 Sample Storage

All urine samples were stored in a 24-hour urine container, then placed in a cooler box with ice to keep the samples cold. The urine samples were sent to the laboratory (Australian Laboratory Services (ALS)) for analysis immediately or, if not possible, within 24 hours. ALS is an International Organization for Standardization certified (ISO/IEC 17025) laboratory and competent in generating valid results. The laboratory kept the urine containers in a freezer room at 4^{o} Celsius. The urine samples were then analyzed within a week. Prior to analysis, methods validation was performed for assessment of urinary silver and fluoride levels.

The hair samples were secured onto sampling cards and placed in a zip lock bag kept in a locked cabinet at room temperature by the principal investigator. The hair samples were then sent by air-courier to the CTQ laboratory in Canada for analysis. The CTQ specializes in identifying, detecting, and quantifying substances in various biological matrices, particularly blood, urine, hair, nails, and tissues. Furthermore, the laboratory has ISO (ISO/CEI 17025) accreditation of the Standards Council of Canada for human toxicology analysis. As the laboratory had validated this analysis method, no further validation was performed.

3.8 Sample Analysis

3.8.1 Sample Analysis: Fluoride in Urine

The analysis of the urine samples for fluoride concentrations was performed using an ion-selective electrode (Thermo Scientific, Orion Star 214) (Figure 3.6).

Figure 3.6 An ion selective electrode machine
Picture courtesy of the Australian Laboratory Services (ALS)

The process was performed based on the NIOSH Manual of Analytical Methods (CDC, 1994):

1. 1 mL well-mixed urine and 1 mL total ionic strength adjustment buffer II (TISAB II) were added to a 50-mL plastic beaker.

2. A small stirring bar was placed into the beaker and mixed continuously on a magnetic stirrer at room temperature.

3. Electrodes were immersed. The sample was allowed to mix for 2 to 3 min, and the displayed readings were recorded in concentration units on the screen.

4. The electrodes and stirring bar were rinsed thoroughly with distilled water and wiped dry with a tissue before the subsequent sample analysis.

5. External calibration curves were prepared in diluent by spiking with different volumes of 100 ppm fluoride standards (Thermo Scientific, Orion IonPlus, 940907)

The analytical method was fully developed and validated with human samples for monitoring purposes following the Eurachem guideline (B. Magnusson, 2014). The report of method validation was attached in Appendix C. Sample preparation and analysis were carried out in a laboratory specifically designed and dedicated to trace element analysis of fluoride standards (Thermo Scientific, Orion IonPlus, 940907). Internal quality control (IQC) was run after calibration and ensured by analysing the urine sample spiked with different concentrations of fluoride standards (0.5 ppm) by diluting the 100 ppm fluoride standards (Thermo Scientific, Orion Ion Plus, 940907). IQC was run after calibration after every 20th sample and at the end of each analytical sequence.

3.8.2 Sample Analysis: Silver in Hair

All tests were performed using a simple quadrupole inductively coupled plasma mass spectrometry (ICP-MS) instrument from PerkinElmer (model NexION 300S, see Figure 3.7) with an autosampler ESI-SC-2 and Syngistix software.

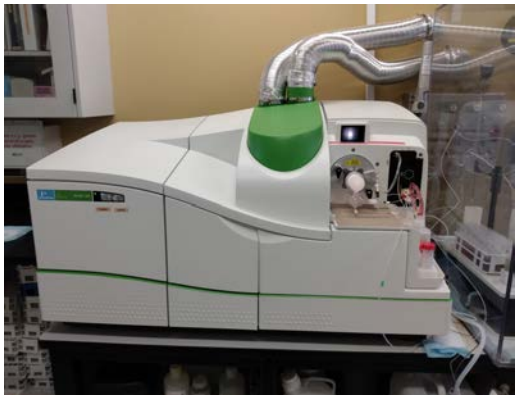


Figure 3.7 The inductively coupled plasma mass spectrometry
Picture courtesy of Centre de toxicologie du Québec (CTQ)

The sample (liquid in general) is pumped into a nebulizer, converted into a fine aerosol using argon gas. A spray chamber separates the small and larger droplets of the aerosol. The small droplets are transported into the plasma torch via an injector. An argon plasma is obtained by the interaction of an intense magnetic field (produced by radiofrequency passing through a copper coil) on a tangential flow of gas at a high flow (15 - 18 L/min). A very high-temperature plasma discharge is thus generated (up to 10,000 K), which allows a sample to decompose into its atomic components that are further ionized into positively charged ions. These ions are transferred via the interface region (cones and extractions lens) to the mass filter (generally a mass filter, usually quadrupole, rarely a sector field), where they are separated according to their mass to charge ratio (m/z). Finally, an ion detector counts the ions emerging from the quadrupole and converts them into an electrical signal.

Analytical method (sample collection, preparation, and analysis):

1. Hair samples were collected by cutting strands close to the scalp from the vertex region of the head with the proximal ends of the samples identified.

2. Hair samples were neither washed nor rinsed prior to sample analysis.

3. A 3-cm hair segment was digested overnight in pressurized Teflon bombs at 120^{o} C under acidic conditions (concentrated OmniTrace nitric acid). The digest was then diluted 20-fold with a diluent containing 0.002% (v/v) cysteine, 100 µg/L (v/v) gold and 5% OmniTrace nitric acid.

4. The diluted samples were analyzed for silver (^{107}Ag) by ICP-MS.

The analytical method was fully developed and validated with human samples for monitoring purposes following 17025 ISO/CEI guidelines. Sample preparation and analysis were carried out in a laboratory specifically designed and dedicated to trace element analysis. Internal quality control (IQC) was ensured by analyzing certified reference materials using NIES 13 (National Institute for Environmental Studies, Japan) and ERM-DB001 (Institute for Reference Materials and Measurements, European Commission) as well as non-certified materials from the Quebec Multielement External Quality Assessment Scheme (QMEQAS) (QM-H-Q1209, QM-H-Q1418, and QM-H-Q1604). IQC was run after calibration after every 10^{th} sample and at the end of each analytical sequence. The analytical method's external quality control (EQC) was assessed by participating in QMEQAS (Canada). Approximately 60 laboratories were involved in this program.

3.8.3 Sample Analysis: Silver in Urine

The analysis of silver levels in urine was performed by the ALS laboratory as well, based on (Lin et al., 2019). Procedure:

1. The volumes of collected urine samples were measured.
2. For each urine sample, 1 mL was diluted with nitric acid to 5 mL with a final concentration of 10% nitric acid.
3. The prepared samples were analysed using an Agilent 7900 ICP-MS (Agilent Technologies, Santa Clara, CA), as shown in Figure 3.8.

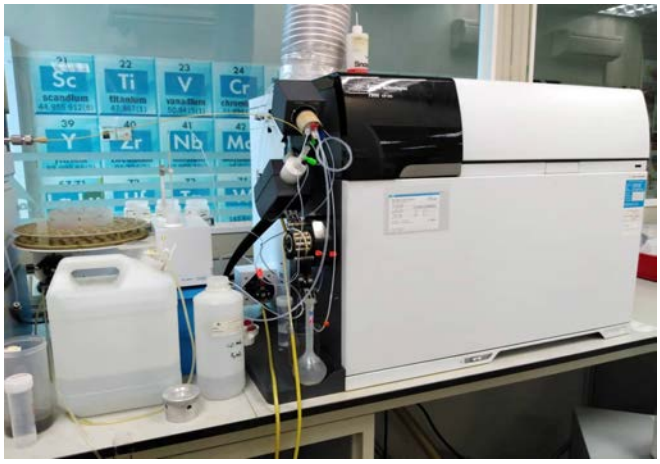


Figure 3.8 The inductively coupled plasma mass spectrometry
Picture courtesy of Australian Lab Service (ALS)

4. Calibration curves were prepared in diluent by spiking with different volumes of 1000 mg/L silver standards solution (Absolute Standards, Inc.; 50% HNO_3, #111-7060).
5. Silver results, expressed in concentration units of µg/L, represented the total metal content of the analysed elements.

The analytical method was fully developed and validated with human samples for monitoring purposes following Eurachem guidelines (B. Magnusson, 2014). The report of method validation was attached in Appendix D. Sample preparation and analysis were carried out in a laboratory specifically designed and dedicated to trace element analysis. Internal quality control (IQC) was ensured by analysing reference materials of silver in urine from Quebec Centre De Toxicologie INSPQ (QM-U-Q1906). IQC was run after calibration, every 10^{th} sample, and at the end of each analytical sequence.

3.9 Data Cleaning

Data cleaning was performed to confirm the completeness of 24-hour urine collection (Appendices E, G, I). The criteria for cleaning data were urine creatinine (mg/kg body weight/day) (World Health Organization, 2014). The indicators of the incomplete collection are urine samples with <8 mg creatinine/kg body weight/day, while urine samples with >22 mg creatinine/kg body weight/day indicate over collection. The range of creatinine in the urine samples was 8 - 22 mg/kg body weight/day which is considered appropriate for children aged 1 - 11 years (World Health Organization, 2014). Study participants whose urine creatinine fell outside the range of 8 – 22 mg/kg body weight/day were excluded for analysis of urinary fluoride. Analysis of urinary silver and hair silver levels were not affected by data cleaning.

3.10 Statistical Analysis

Statistical analysis was performed using IBM SPSS (Statistical Package for the Social Science) software, version 26. For descriptive statistics, mean, standard deviation, median, and interquartile range were used while Fleiss multirater kappa, Pearson and Spearman correlation tests, and Friedman's test were used for inferential statistics.

Only nine subjects were included for the data analysis as six had under- or over-collection of 24-hour urine. The data for urinary fluoride levels at baseline, first, and second 24-hour urine samples were tested for normality.

Fleiss' kappa, κ (Fleiss, 1971; Fleiss et al., 2003), is a measure of inter-rater agreement used to determine the level of agreement between two or more raters (also known as "judges" or "observers") when the method of assessment, known as the response variable, is measured on a categorical scale. In the present study, Fleiss multirater kappa was used to determine if there was agreement among three investigators

on whether the examined teeth (N=193) were sound, presented with first visual changes in enamel, had distinct visual changes in the enamel, localized enamel breakdown with no visible dentin, underlying dark shadows from dentin, distinct cavity with visible dentin, extensive distinct cavity with visible dentin or with the presence of restoration.

Pearson's and Spearman's product-moment correlations were performed to examine bivariate linear relationships and determine any significant relationships between the two variables. Independent variables comprising age, body mass index (BMI), number of teeth treated by SDF, amount of applied SDF, and dependent variables were baseline urinary fluoride levels (baseline), urinary fluoride levels in the first (F1), and the second (F2) 24 hours after SDF. The correlation helps to clarify how the variables are related in strength and magnitude. The Pearson and Spearman correlation coefficient, r, is utilized to measure the extent of a linear relationship. r can only take on values from -1 to +1. This study used the Guildford and Fruchter (1973) rule of thumb to determine the strength of the relationship between variables. The p-value was set at .05 in these analyses. Table 4.9 in Chapter 4 shows the criteria for interpreting the strength of the relationship between two variables.

The Friedman test was used to examine the difference between groups of urinary fluoride levels collected at baseline and following the first and second 24 hours statistically.

CHAPTER 4: RESULTS

4.1 Demographics Data

Table 4.1 shows the demographics data. The subjects comprised 15 children aged between 57 to 110 months at baseline with a mean ±SD of 78 ±19 months. The majority were female and of Malay ethnicity. In total, 61 teeth received SDF treatment, 62.3% of them primary molars. On average, each child had four teeth treated with SDF. The number of teeth treated by SDF ranged from 2 to 8 per child. The mean ± SD of applied SDF for each child was 42.3 ± 9.7 mg of SDF. A mean of 44.2 to 48.07µL of SDF was applied per child, depending on the relative density of ammonia.

Table 4.1 Demographics of participants

Gender (N=15)	Male (%) 5 (33.3)	Female (%) 10 (66.7)
Ethnicity (N=15)	Malay (%) 10 (66.7)	Chinese (%) 5 (33.3)
Age of children [A] (N=15)	78 months ± 19 months (57 – 110 months)	
Total number of SDF-treated-teeth for 15 children	61	
Tooth type per SDF treatment (N=61)	Anterior 23 (37.7%)	Molar 38 (62.3%)
Number of teeth treated with SDF per child [A]	4 ± 2 (2 – 8)	
SDF applied (mg) [A]	42.3 ± 9.7 (26.7 – 64)	
Calculated volume of SDF applied (µL), based on relative density of ammonia (0.880) in SDF (Riva Star®) [A, B]	48.07 ± 11.06 (30.34 – 73.18)	

Table 4.1 Continued

Calculated volume of SDF applied (μL) based on relative density of ammonia (0.957) in SDF (Riva Star®) [A, B]	44.20 ± 10.17 (27.90 – 67.29)

[A] Mean ± standard deviation (range)

[B] Specific gravity of ammonia in Silver Diamine Fluoride (Riva Star®): 0.880 – 0.957

4.2 Inter-rater Reliability Test

Fleiss' multirater kappa (Table 4.2) showed κ=.725 (95% CI, .723 to .727), $p < .001$. There are no rules of thumb to assess how good the kappa value of 0.725 is. The classification of Cohen's kappa (Table 4.3) has been suggested to assess how good the strength of agreement is based on the value of Cohen's kappa coefficient**.** Based on this classification, Fleiss's kappa (κ)= 0.725 represents good strength of agreement between three investigators on the caries detection.

Table 4.2 Fleiss multirater kappa

	Kappa	SE	P value	95% CI	
				LB	UB
Overall agreement	0.725	0.026	0.001	0.723	0.727

Table 4.3 Classification of Cohen's kappa

Value of κ	Strength of Agreement
< 0.20	Poor
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Good
0.81-1.00	Very good

4.3 Fluoride Levels in Urine

4.3.1 The Results for Methods Validation of Fluoride in Urine

The method used to analyze the fluoride levels in urine samples was validated for this study and found to be suitable for use by the laboratory. The method was based on the NIOSH Manual of Analytical Methods (CDC, 1994). Table 4.4 shows some validation parameters for this method. The average % recovery for Matrix Spike Samples was 92 % to 101 %, which falls within the acceptable limit of 80 to 120%. The RSD of repeatability and reproducibility were 2.207 % and 1.542%, respectively. The correlation coefficient for the calibration curve of fluoride in urine was > 0.995; hence the calibration was linear.

Table 4.4 Results for methods validation of urinary fluoride

Analytes	LOD (mg/L)	LOQ (mg/L)	Repeatability (%)	Reproducibility (%)	Recovery (%)	Correlation coefficient (r^2)
Fluoride	0.003	0.01	2.207	1.542	92-101	> 0.999

LOD: Limit of Detection; LOQ: Limit of Quantification

4.3.2 Data Cleaning

After data cleaning, six subjects were excluded from the analysis of the results (Appendices E, G, I), leaving only nine participants. Out of nine participants, one participant did not collect the second 24-hour urine sample following SDF treatment. Hence, only eight participants were included for the data analysis of urinary fluoride levels.

4.3.3 Normality Tests

Table 4.5 Normality test

Variables	Shapiro-Wilk	
	df	Sig.
Age	8	.262
Body mass index (BMI)	8	.186
Number of teeth treated by SDF	8	.004
Number of surface of teeth treated by SDF	8	.446
Amount of applied SDF	8	.473
Baseline urinary fluoride level	8	.658
Urinary fluoride levels in the first 24-hours after SDF (F1)	8	.012
Urinary fluoride levels in the second 24-hours after SDF (F2)	8	.536

Based on the normality tests for all variables (Table 4.5), the null hypothesis of normal distribution for data on the number of teeth treated by SDF and urinary fluoride levels in the first 24 hours (F1) after SDF were rejected as the p-value < 0.05. For data on age, BMI, number of the surface of teeth treated by SDF, amount of applied SDF, baseline urinary fluoride levels, and urinary fluoride levels in the second 24 hours (F2) after SDF, the null hypotheses of normality were retained as $p > 0.05$.

The ratio of skewness and kurtosis was used to further assess the normality of data (Table 4.6). It was found that only data on urinary fluoride levels in the first 24 hours after SDF was not distributed normally. This is because the ratio of both skewness and kurtosis for this data was outside the -1.96 to 1.96 range (Kim, 2013).

Table 4.6 Descriptive Statistics and Ratio of Skewness, Kurtosis

Variables		Statistic	Std. Error	Statistic/Std. Error
Age	Skewness	0.678	0.752	0.902
	Kurtosis	-0.701	1.481	-0.473
BMI	Skewness	1.132	0.752	1.507
	Kurtosis	0.957	1.481	0.647
Number of teeth treated with SDF	Skewness	1.170	0.752	1.556
	Kurtosis	-0.476	1.481	-0.321
Number of surface of teeth treated with SDF	Skewness	0.402	0.752	0.535
	Kurtosis	-1.231	1.481	-0.831
Amount of SDF applied	Skewness	0.913	0.752	1.214
	Kurtosis	1.178	1.481	0.795
Baseline urinary fluoride level	Skewness	0.627	0.752	0.834
	Kurtosis	0.184	1.481	0.124
F1	Skewness	2.034	0.752	2.705
	Kurtosis	4.508	1.481	3.044
F2	Skewness	0.928	0.752	1.234
	Kurtosis	1.042	1.481	0.704

F1- Urinary fluoride level in the first 24 hours following SDF treatment

F2- Urinary fluoride level in the second 24 hours following SDF treatment

4.3.4 The Mean, Standard Deviation, Median, and Interquartile Range of Urinary Fluoride Levels

The mean, standard deviation, median, and interquartile range of urinary fluoride at the three different time points are shown in Table 4.7. The complete data of urinary fluoride levels at baseline, F1, and F2 are in Appendices F, H, J.

Table 4.7 Mean, standard deviation, median and interquartile range of urinary fluoride levels (µg/24-hour) at baseline, and first and second 24-hours after SDF treatment

Analytes (N=24)	Mean	Standard Deviation	Median	Interquartile Range
Baseline urinary fluoride level	481.41	241.59	498.33	381
F1	673.80	329.82	574.92	318.87
F2	487.21	244.31	474.35	324.21

F1- Urinary fluoride level in the first 24 hours following SDF treatment

F2- Urinary fluoride level in the second 24 hours following SDF treatment

4.3.5 Pearson and Spearman Correlation Tests

Part of the objectives of this study was to ascertain fluoride levels in the urine samples. In order to determine if this outcome was influenced by factors like age, BMI, number of teeth treated with SDF, number of the surface of teeth treated with SDF and amount of SDF applied, Pearson and Spearman correlation coefficient analyses were used. As depicted in Table 4.8 and table 4.9, there was significant high correlation between baseline and F1 (r= .883, p= .002) as well as between baseline and F2 (r= .733, p= .039) but no significant correlation between age, BMI, number of teeth treated with SDF, number of surface of teeth treated with SDF and SDF amounts applied, and urinary fluoride level outcomes. There was a significantly high correlation between the number of teeth treated with SDF and the number of the surface of teeth treated with SDF (r= .867, p= .002).

Table 4.8 Correlation between demographic and clinical characteristics with urinary fluoride levels

Pearson and Spearman Correlations									
		Age	BMI	Number of SDF-treated teeth	Number of surface of teeth treated by SDF	Amount of applied SDF	Baseline	F1	F2
Age	Pearson Correlation	1							
	P value								

BMI	Pearson Correlation	.141	1						
	P value	.717							
Number of SDF-treated teeth	Pearson Correlation	-.322	-.596	1					
	P value	.397	.090						
Number of surface of teeth treated by SDF	Pearson Correlation	.034	-.465	.867**	1				
	P value	.932	.208	.002					
Amount of applied SDF	Pearson Correlation	-.298	-.274	.495	.361	1			
	P value	.436	.475	.175	.339				
Baseline	Pearson Correlation	-.099	.223	-.182	.036	-.392	1		
	P value	.801	.564	.640	.926	.297			
F1	Spearman's rho Correlation Coefficient	-.159	.650	-.167	.000	-.433	.883**	1	
	P value	.683	.058	.668	1	.244	.002		
F2	Pearson Correlation	.205	.523	-.201	.119	-.448	.733*	.558	1
	P value	.627	.184	.634	.780	.265	.039	.151	

**Correlation significant at the 0.01 level (2-tailed)

* Correlation significant at the 0.05 level (2-tailed)

F1- Urinary fluoride level in the first 24 hours following SDF treatment

F2- Urinary fluoride level in the second 24 hours following SDF treatment

Table 4.9 Guildford rule-of-thumb table

r	Strength of Relationship
<.20	Slight relationship
.20-.40	Low correlation, definite but small
.40-.70	Moderate correlation, substantial relationship
.70-.90	High correlation, marked relationship
>.90	Very high correlation, very dependable relationship

Source: Guildford Rule of Thumb (1973

4.3.6 Friedman's Test

The Related-Samples Friedman's Two-Way Analysis of Variance by Ranks (Friedman's test), a non-parametric alternative to the one-way ANOVA with repeated measures, was used to test the difference in urinary fluoride levels collected at baseline and the first and second 24 hours after SDF treatment. As seen in Table 4.10, the results show that the differences were statistically significant (p-value= 0.008). Hence, the distribution of urinary fluoride levels at baseline, F1, and F2 were not the same. The pairwise comparisons (Table 4.11) showed significant differences in urinary fluoride levels taken at baseline and the first 24 hours after SDF treatment. The null hypothesis stated that SDF PK characteristics (fluoride) measured in their biomarkers (urine) taken before and after SDF treatment was the same. Therefore, the null hypothesis was rejected.

Table 4.10 Related samples of Friedman's Two-Way Analysis of Variance by Ranks

Total N	Test Statistics	Degree of freedom	p-value
8	9.75	2	0.008

Table 4.11 Pairwise Comparisons

	Pairwise Comparisons		
Sample 1- Sample 2	Test Statistics	Std. Error	p-value
Baseline - F2	-3.75	.500	1.000
Baseline - F1	-1.500	.500	0.008
F2 - F1	1.125	.500	0.073

F1- Urinary fluoride level in the first 24 hours following SDF treatment

F2- Urinary fluoride level in the second 24 hours following SDF treatment

Each row tests the null hypothesis that Sample 1 and Sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05. The significance values were adjusted by the Bonferroni correction for multiple tests.

4.3.7 The Observed Power of Study

Based on posthoc power analysis, the observed power of the study is 79.3% given sample size (N=8), effect size (1.183417), and alpha error probability (0.05). The effect size is calculated based on the mean and standard deviation of urinary fluoride levels taken at baseline and the first 24 hours after SDF treatment, with the correlation between groups (r= 0.883). The analysis was performed using the G*Power software, as depicted in Appendix O.

4.3.8 Daily Urinary Fluoride Excretion (DUFE) and Corresponding Total Daily Fluoride Intake Levels (TDFI)

Table 4.12 shows the daily urinary fluoride excretion (DUFE) level calculated based on the total daily fluoride intake (TDFI) of 0.1 mg/kg body weight (World Health Organization, 2014). Comparisons were made with actual daily urinary fluoride excretion (DUFE) levels from the subjects in this study taken at baseline and the first and second 24 hours after SDF

application. The result showed that subjects 2, 5, and 11 had DUFE corresponding to higher TDFI for 0.1 mg/kg body weight.

Table 4.12 Fluoride levels in urine (TDFI and DUFE)

Subject	Body weight	TDFI: 0.1mg/kg body weight	Calculated DUFE (mg/24h)	Actual DUFE (mg/24h)		
			[A] Based on TDFI 0.1mg/ kg BW	Baseline	F1	F2
1	17.1	1.71	0.6	0.6	0.5	NA
2	25	2.5	0.9	0.9	1.4	0.6
3	22	2.2	0.8	0.3	0.5	0.3
4	13.8	1.38	0.5	0.2	0.4	0.3
5	15.6	1.56	0.6	0.4	0.9	0.2
6	30	3	1.1	0.7	0.9	1.0
7	21.4	2.14	0.8	0.3	0.6	0.1
8	16.5	1.65	0.6	0.2	0.5	0.2
9	13	1.3	0.5	0.2	0.3	0.3
10	17.3	1.73	0.6	0.2	0.4	0.2
11	13	1.3	0.5	0.5	0.6	0.5
12	27.2	2.72	1.0	0.3	0.3	0.2
13	14.3	1.43	0.5	0.4	0.3	0.4
14	16.6	1.66	0.6	0.5	0.6	0.4
15	30	3	1.1	0.5	0.7	0.6

DUFE: Daily Urinary Fluoride Excretion (DUFE: TDFI x 0.35 + 0.03)

TDFI: Total Daily Fluoride Intake

F1- Urinary fluoride level in the first 24 hours following SDF treatment

F2- Urinary fluoride level in the second 24 hours following SDF treatment

4.4 Silver Levels in Urine

The method for analyzing silver levels in urine samples was validated for this study and found suitable for use by the laboratory. The method used was based on Lin et al., (2019) followed by analysis by Inductive Coupled Plasma- Mass Spectrometry(U.S EPA 6020 A). A summary of the result is shown in Table 4.13. The average % recovery for Matrix Spike Samples was 100 %, which falls within the acceptable limit of 80 to 120%. The RSD of repeatability and reproducibility was 7.95 % and 6.27%, respectively. The correlation coefficient for the calibration curve of silver in urine was > 0.995; hence the calibration was linear.

Table 4.13 Results for methods validation of urinary silver

Analyte	LOD (µg/L)	LOQ (µg/L)	Repeatability (%)	Reproducibility (%)	Recovery (%)	Correlation coefficient (r^2)
Silver	0.003	0.01	7.95	6.27	100	>0.999

Table 4.14 showed the range of silver levels in the urine samples of the 15 subjects, all of which were at <1.0µg/24 hours before and after the second 24 hours after SDF treatment. Meanwhile, in the first 24 hours after SDF treatment, all subjects had silver <1.0 µg/24 hours except for one whose silver level was 2.0 µg/24 hours (Appendix K, L, M).

Table 4.14 Metals in biofluids (µg)/24 hours

Time points	Range of silver (µg/24 hours)	Range of silver (µg/L) (N= 45)
Before SDF treatment	< 1.0	< 0.05 - 1
First 24 hours after SDF treatment	1.0 - 2.0	< 0.05 - 2.64
Second 24 hours after SDF treatment	<1.0	< 0.05 - 1.04

4.5 Silver Levels in Hair

The method to analyse silver level in hair samples was previously validated by the CTQ laboratory. The validation parameters for the analytical method used for Ag analysis in hair samples are presented in Table 4.15. The average % recovery for Matrix Spiked Samples was 89 – 116 % which falls within the acceptable limit of 80 to 120%. The relative standard deviation of repeatability and reproducibility were 3.3% and 4%, respectively. The correlation coefficient for the calibration curve of silver in hair was > 0.995; hence the calibration was linear.

Table 4.15 Results for methods validation of silver levels in hair samples

Analyte	LOD (µg/g)	LOQ (µg/g)	Repeatability (%)	Reproducibility (%)	Recovery (%)	Correlation coefficient (r^2)
Silver	0.2	0.6	3.3	4.4	89 - 116	> 0.999

Table 4.16 shows the range of silver (µg)/g in the hair samples of 15 subjects. Seven hair samples were collected from each subject at different time points. The range of hair silver levels after SDF treatment at day 7, 14, 30, 60, 75, and 90 (<0.2 - 0.69 µg/g) were found to fluctuate around the baseline levels (<0.2 – 1.7 µg/g).

Table 4.16 Silver levels in hair (µg/g)

Time points		Range of silver in hair (µg/g) (N=105)
Before SDF treatment		< 0.2 - 1.7
After SDF treatment	Day 7	< 0.2 - 0.63
	Day 14	< 0.2 - 0.69
	Day 30	<0.2 - 0.6
	Day 60	<0.2 - 0.63
	Day 75	<0.2 - 0.5
	Day 90	<0.2 - 0.5

CHAPTER 5: DISCUSSION

This study assessed the pharmacokinetic (PK) of silver diamine fluoride by determining silver and fluoride levels in urine samples and silver levels in hair samples before and after SDF application. The number of teeth treated with SDF was not predetermined, as the aim was to simulate normal clinical situations in which a patient may present various numbers of decayed teeth. A variety of study designs are available for addressing the knowledge gap in the under-researched topic of PK of silver diamine fluoride in children.

Different study designs in assessing PK characteristics include individual PK studies and population- and physiologically based PK modelling. Individual PK approaches are best when rapid turnaround of PK parameters is needed or in defining complete individual PK profiles (Banks, 2018). These analyses require rich concentration-time data and typically employ noncompartmental methods. Population-based PK (popPK) modelling for supporting clinical trials can reduce the number of samples required from each individual within a population by increasing the overall population size. In contrast to individual PK analysis, popPK modeling approaches rely on concentration-time data from multiple individuals and often utilize pooled data from more than one study (Banks, 2018). PopPK methods can, and often are, applied to rich data that has been collected. However, its real value lies in analyzing clinical data collected in settings where it is not practical to do so for rich data, such as in Phase 2 and 3 trials (Banks, 2018) involving a few hundreds or thousands of individuals, respectively. PopPK tends to use rather complex mathematical and compartmental methods to reach conclusions. Because of technical constraints in popPK model development and optimization, popPK approaches often take considerably longer time (Banks, 2018).

Physiologically based pharmacokinetic (PBPK) models have been developed that incorporate paediatric developmental physiology to predict drug exposure in children based on

existing clinical data from adults. The limitations of PBPK models are well documented, including the appropriate validation (mainly statistical) needed for them (Krekels et al., 2011; Tod et al., 2008). Recently, a physiologically based pharmacokinetic (PBPK) model was developed to predict silver disposition in children following SDF treatment. The simulation in children suggests that conventional SDF applications to teeth result in lower plasma and tissue silver concentrations than toxic concentrations (Chen et al., 2020).

Biomarkers for silver and fluoride are relevant in assessing the exposure of silver diamine fluoride in the body. Different biomarkers are available, e.g., blood, urine, and hair sampling. Each biomarker is appropriate for a specific research question. The integration of both biomarkers is sometimes recommended to mitigate the weakness of using each biomarker alone. However, the choice of biomarkers in the paediatric population is unique, with ethical concerns. This is especially obvious in blood-drawing in children solely for research purposes (National Institutes of Health Malaysia, 2011). Hence, urine and hair were chosen as the biomarkers in this study.

SDF applied in this study averaged 42.3 mg ± SD: 0.7, or four teeth per child. The applied SDF amount was about three to ten times more than in earlier studies, where the estimated amounts of SDF applied were 4 - 11 mg for five teeth (Lin et al., 2019) and 7.57 mg for three teeth (Vasquez et al., 2012). The variation of the amount of applied SDF may be due to the usage of SDF in capsule form rather than the bottle form. Other possible factors (Crystal et al., 2019) include the temperature of the clinic environment, different physical properties of Riva Star® compared to the Saforide® studied by (Vasquez et al., 2012) and Advantage Arrest® studied by (Lin et al., 2019).

According to an *in-vitro* study by (Crystal et al., 2019), a single drop of 38% SDF (Advantage Arrest) contains an average weight of 40 mg with a mean volume of 32.55 µL,

which is 30% more than the 25 µL reported by the manufacturer. In this study, as instructed by the manufacturer, 38% SDF (Riva Star®) in a single capsule was used per treatment visit. The mean and standard deviation of SDF amounts applied (42.3 ± 9.7 mg) was close to that of SDF per drop (40 mg) reported by Crystal et al. (2019). On the other hand, 44.2 µL to 48.07 µL were the average volumes of SDF applied in this study which is close to the 50 µL (per capsule) reported by the manufacturer of Riva Star®.

The U.S EPA states that twenty-five grams of silver per lifetime exposure should be avoided to reduce the risk of argyria (U.S Environmental Protection Agency, 2003). This study shows that the highest dose of applied SDF per child is 64 mg, for seven teeth. This translates to 390 applications of such dosage per lifetime to lead to argyria, assuming other possible sources of silver from the environment are excluded. The actual number of applications of Riva Star® that could lead to argyria is estimated to be more than 390 applications because the exact concentrations of silver in Riva Star® are unknown. This finding was similar to 400 SDF applications required to cause argyria as reported by (Vasquez et al., 2012), based on the highest dose of applied SDF (2.37 mg), with one gram of silver per lifetime exposure as the limit. Twenty-five grams of silver per lifetime was used as the limit in the current study, as it's the oral dosage of silver associating with an increased risk of argyria. In comparison, one gram of silver per lifetime was the limit for an intravenous route. As SDF application is recommended for carious teeth on a six-month basis (Seifo et al., 2020), applications up to 390 times per lifetime are not clinically relevant.

To assess the risks of an overdose of fluoride, estimating its intake is necessary. However, the process of measuring fluoride intake is expensive and technically demanding. Measurement of urinary fluoride is possible to determine the fluoride intake (Villa et al., 2010). In this study, the range of reported urinary fluoride concentrations for all subjects (except subjects 2, 5, and 11) was lower than daily urinary fluoride excretion (DUFE) corresponding

to a high total dietary fluoride intake (TDFI) of 0.1 mg/kg body weight. The higher levels of DUFE for these three subjects were from the first 24 hours after SDF treatment, while their baseline and second 24-hour fluoride levels were lower than the DUFE corresponding with TDFI 0.1mg/kg BW. A TDFI of 0.1 mg/kg BW is the upper limit for risk of dental fluorosis in children aged 1 - 8 years (European Food Safety, 2005). A 0.1mg of fluoride intake per kg body weight is equivalent to 2.5 mg of fluoride per day for children aged 4 - 8 years, which is the age group for subjects 2, 5, and 11. The estimated optimal fluoride level for this age group is only 1 mg per day (European Food Safety, 2005). Generally, the results of this study show that fluoride levels excreted through urine at the first 24 hours after SDF treatment were significantly higher than before SDF treatment (p-value 0.008). Although three of the fifteen subjects (20%) had high total daily fluoride intakes from SDF application, this may not be clinically significant as SDF is not recommended as a daily application.

The mean ± SD of fluoride levels recovered in urine was 0.412 ± 0.197 mg/24-hour for the baseline, 0.538 ± 0.271 mg/24-hour for the first 24 hours, and 0.38 ± 0.21 mg/24-hour for the second 24 hours after SDF treatment (Appendix F, H, J). In (Lin et al., 2019), 1.29 ± 0.81 mg/24-hour fluoride was recovered in the first 24 hours after SDF treatment for sixteen adults. The recovered urinary fluoride in this study was less than half that in the predecessor study (Lin et al., 2019), although the average amount of applied SDF was higher. This is assumed to be due to the different age groups of participants, as the proportion of fluoride excreted in urine is influenced by age. According to the WHO, an increase in body weight for older age group individuals results in an increased DUFE corresponding to TDFI. For this study, SDF application did not result in an increase of DUFE corresponding to high TDFI, except in three subjects. The fluoride is absorbed through the gastrointestinal tract before entering the systemic circulation upon topical application of SDF. As fluoride concentrations in the plasma were not evaluated in this study due to ethical concerns, the fluoride absorbed into the systemic

circulation is unknown. Lin et al. (2019) reported that following SDF treatment, serum fluoride levels fluctuate around the baseline level, and this fluctuation is not related to SDF application. They concluded that low amounts of fluoride from SDF were being absorbed into the systemic circulation. On the other hand, Sari and colleagues measured urinary fluoride levels in four young adults who received SDF treatment. Urine samples were collected at baseline, and controlled time of 30 minutes, two and three hours after SDF treatment. They reported that the maximum average urinary fluoride level (0.216 mg/L) was found three hours after SDF treatment. The current study involved 24-hour urinary collections. Results showed an average of 0.947 mg/L of urinary fluoride levels at baseline, then increased to 1.027 mg/L in the first 24-hour after SDF treatment and dropped to 0.916 mg/L in the next 24-hour. The highest average urinary fluoride level in Sari and colleagues' study was lower than the average baseline urinary fluoride levels of the present study. The World Health Organization recommended the use of 24-hour urine collection wherever possible. When 24-hour urine collections are not feasible, a time-controlled method could be used to provide estimations of 24-hour urine collections (World Health Organization, 2014). The authors could not account for the differences in urinary fluoride levels in the present study and Sari et al. 2017.

Silver is mainly excreted via feces, but measurements of urinary silver may provide a convenient index of silver absorption by all routes and serve as a guide to the total silver content of a body at blood levels of $<100\ \mu g \cdot L^{-1}$ (Wan et al., 1991). At higher concentrations of silver, the pattern of excretion is irregular (Lansdown, 2010). In a study on 2 to 17-year-olds in Germany, Hteitland and Köster (2006) reported that the upper limit for normal ranges of the urinary silver level was 0.05 µg/L. In this study, 4 of 15 subjects before SDF treatment, and 13 of 15 subjects in the first 24 hours after SDF treatment, and 11 of 14 subjects in the second 24 hours after SDF treatment had urinary silver levels above 0.05 µg/L (Appendices K, L and M). About 79 - 87% of the subjects had urinary silver levels higher than 0.05 µg/L after SDF

treatment, compared to only 27% before treatment. The range of urinary silver levels before and after the second 24 hours after SDF treatment was about <0.05-1 µg/L, whereas <0.05-2.6 µg/L of urinary silver was measured in the first 24 hours after treatment. The maximum urinary silver level in the first 24-hours after SDF treatment was two-fold higher than the baseline and first 24 hour results. However, as the reported silver levels were in range, statistical tests were not possible.

In the United States, Lin et al. (2019) reported that urinary silver levels were in the range of <0.05-0.06 µg/L before and after SDF treatment. In comparison, the recovered urinary silver levels after SDF treatment in the present study reached 2.64 µg/L, which was much higher than <0.05-0.06µg/L. In Africa, Godebo et al. (2019) reported the maximum value of 0.2 µg/L of urinary silver levels in their studied adult and children populations. The studied subjects live in a rural region with geologic rifting and volcanic activities, and they are dependent on groundwater wells as the primary water source. This condition leads to long-term exposure to the naturally occurring toxic and essential elements from water and food sources. Compared to these studies, the upper limit of baseline urinary silver levels found in this study (1 µg/L) was higher. The authors could not account for the differences in baseline urinary silver levels as silver levels in food and water sources may vary in different geographical regions. It is known that silver is found in food, drinking water, and soil. Furthermore, the absorption of silver levels in the human body varies widely according to age, health and nutritional status, and diet composition (ATSDR, 1990).

The LOD for urinary fluoride and urinary silver in this study were 0.003 mg/L and 0.003 µg/L, respectively, lower than the 0.01 mg/L and 0.05 µg/L reported by Lin et al. (2019). This translates into achieving a lower concentration of silver-level that is distinguishable from method blank results.

Following SDF treatment, the hair silver levels in this study were found to fluctuate around the baseline level. The baseline silver level ranged between <0.2-1.7 µg/g, while after SDF treatment, it ranged between <0.2- 0.69 µg/g. The fluctuation appeared to be unrelated to the SDF applications, as the hair silver level taken after the SDF treatment did not exceed the baseline levels. The half-life of silver is estimated to be 46 ± 26 hours (Lin et al., 2019). Therefore, the measurement of silver levels in hair for up to 90 days in this study is sufficient to determine silver concentrations. Furthermore, the LOD of 0.2 µg/g is considered sufficient as the mean silver level in hair samples of children from the Kingdom of Saudi Arabia and the United States studies were 0.39 µg/g and 2.13 µg/g, respectively (Table 2.5). As most of the hair silver levels were below the limits of detection, it was not possible to calculate the pharmacokinetic data by plotting the silver concentrations in hair samples (µg/g) against the corresponding time of hair collection (baseline, and days 7, 14, 30, 60, 75, and 90 after SDF treatment).

A recent large-scale study aimed at developing and validating quantifying methods in measuring metal elements in hair noted that normal silver levels in hair ranged between 0.02-1.31 µg/g in healthy volunteers (Goullé et al., 2005). One participant in this study had a baseline silver level above 1.31 µg/g (Appendix N), while the rest had levels below that. This participant was an outlier in the hair silver level measurement. The slightly higher silver level may not be clinically significant, as the average hair silver levels among subjects with occupational exposure to silver were 130 µg/g, with evidence of silver deposition in the outer hair layers (DiVincenzo et al., 1985).

Regarding adverse effects following SDF applications, none of the participants reported any pain/discomfort, nausea, and vomiting, nor was any incidence of gingival bleaching or swelling observed. However, all the participants' teeth were stained black following the treatment, and all SDF-treated teeth showed arrested caries lesions when reviewed on day 30.

In the current study, several study limitations involving bias and imprecision existed. There was selection bias due to the limited ethnicity of study participants. The majority of included participants were Malay children with minority Chinese children. As race affects inter-and intra-patient pharmacokinetics, it was crucial to include children of different ethnic groups such as Indians and natives in peninsular Malaysia. Imprecision of the current study was due to the small sample size (N=8) included for analysis of urinary fluorides. The remaining 7 study participants were not included in data analysis due to under-or overcollection of 24-hour urines and lack of urine collection (second 24-hour after SDF). Inaccurate 24-hour urine collection was a common issue among children. Up to 80% of children under 18 years old submitted inaccurate urine collection samples for analysis, according to a study by Chan et al. 2019. No parental or child factors were associated with inadequate collection. Education of patients and parents regarding the importance of the research and frequent reminder on the correct urine collection method was suggested to improve the adherence to instruction for 24-hour urine collection. The small sample size (N=8) had led to lower observed power of the study, 79.3%, which was near to the adequate statistical power of 80% (Di Stefano, 2003). It was recommended to increase the number of participants to enhance the study's findings and increase the study power (Miler & Simundić, 2013).

Due to the usage of non-probability sampling methods (convenience sampling), the external validation (generalizability) of results was limited to the population that shared similar characteristics with the sample, which was healthy Malay and Chinese children aged between 57-110 months residing in Malaysia (Ferguson, 2004). For future research, it is recommended to use probability sampling to counters selection bias. In regards to internal validity, this study was valid as the SDF treatment and response variables (urinary silver and fluoride; hair silver levels) changed together, the SDF treatment precedes changes in the response variables, and there were no confounding factors able to explain the study results (Ferguson, 2004). Besides,

the reporting of current research was transparent with complete study information. Reporting guidelines for clinical PK studies were used as guidance in reporting (Kanji et al., 2015).

Future study with measurement of silver and fluoride levels in the freshly opened SDF capsule and days after the opening, with measurements of pharmacokinetics characteristics of SDF, is recommended. The recommendation is based on the results of existing studies proving different silver and fluoride concentrations than the manufacturer's claims and the instability of these compounds.

CHAPTER 6: CONCLUSION

In this study, SDF treatment was provided for 15 children. Urinary silver, fluoride levels, and hair silver levels were determined before and after SDF treatment for all children. The author reported that:

1. The range of urinary silver levels taken at baseline, first and second 24-hour after SDF treatment was <1µg/24-hour, 1-2 µg/24-hour, and <1 µg/24-hour, respectively. The reported urinary silver levels were extremely low. Urinary silver levels rose to 2µg/24-hour in the first 24-hour after SDF treatment. However, statistical test was not possible to test the difference of urinary silver levels taken at different times as the reported silver levels were in the range.

2. The median and interquartile range of urinary fluoride at baseline, first and second 24 hours after SDF were 498.33 (381) µg/24-hour, 574.92 (318.87) µg/24-hour, and 474.35 (324.21) µg/24-hour, respectively. Statistically, urinary fluoride levels at the first 24 hours after SDF treatment were significantly higher than those at the baseline. In terms of total daily fluoride intake (TDFI), 3 of the 15 participants had daily urinary fluoride excretions corresponding to high TDFI following a single SDF application. The corresponding high TDFI following SDF treatment is not clinically significant, as SDF applications are recommended at six-monthly intervals. No adverse effects were observed among the study participants.

3. The range of hair silver levels taken at day 7, 14, 30, 60, 75, and 90 after SDF treatment (<0.2-0.69µg/g) fluctuated around the baseline levels (<0.2-1.7µg/g). The fluctuation of hair silver levels was not related to the SDF treatment. Calculation of pharmacokinetic data was not possible as most hair silver levels fall below the limit of detection.

Within the limitations of this study, the author concluded that SDF usage is safe among children. However, clinicians should remain vigilant when using SDF in children, as the amount of SDF applied in the present study is three to ten times higher than in earlier studies that investigated the usage of SDF in adults. Variations in the amounts of SDF applied across different studies are major considerations in dosing recommendations for paediatric populations.

The author recommended that clinicians to use SDF as biannual applications, following current clinical guidelines. The clinicians should avoid using SDF as daily applications due to the risk of dental fluorosis, as evidenced by the fact that three out of fifteen participants had a high total daily fluoride intake following SDF applications. Per an individual's lifetime, no more than 390 times SDF applications should be used to avoid the risk of argyria.

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