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Development and validation of a LC-MS/MS method for quantitative analysis of uraemic toxins *p*-cresol sulphate and indoxyl sulphate in saliva

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ABSTRACT

p-Cresol sulphate (pCS) and indoxyl sulphate (IS) are uraemic toxins, the concentration of which in serum correlate with the stage of renal failure. The aim of this study was to develop and validate a high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the analysis of pCS and IS in saliva. This is the first time, to our knowledge, that such a method has been developed using saliva. Unstimulated, fasting saliva was collected from healthy volunteers in the morning and pooled for validation assay. The method was validated for linearity, precision, accuracy, stability (freeze/thaw stability, stability in autosampler, short- and long-term stability, stock solution stability), dilution integrity and matrix effect. The analysed validation criteria were fulfilled. No influence of salivary flow (pCS: $p=0.678$; IS: $p=0.238$) nor type of swab in the Salivette device was detected. Finally, using the novel validated method, the saliva samples of healthy people ($n=70$) of various ages were analysed. We observed a tendency for an increase of concentration of toxins in saliva in the elderly. This could be a result of age-related diseases, e.g., diabetes and kidney function decline. We can conclude that the novel LC-MS/MS method can be used for the determination of pCS and IS in human saliva. The results encourage the validation of saliva as a clinical sample for monitoring toxin levels in organisms.

1. Introduction

p-Cresol sulphate (pCS) and indoxyl sulphate (IS) are uraemic toxins, 90% of which occur in blood plasma in a form bound to proteins and are biologically active. Furthermore, they are physiologically almost completely eliminated by the kidneys. However, in the case of chronic kidney disease, accumulation of these compounds results in metabolic disorders and leads to the condition called uraemia. Concentration of these substances in serum correlates with the stage of renal failure and is related to disease progression and an increased risk of, e.g., cardiovascular disease. Hence, these substances should be constantly monitored to protect patients from the progression of the disease and its negative outcomes. An excellent material for this goal could be saliva [1–4].

Saliva is an alternative diagnostic material to blood, offering several advantages over serum/plasma. First of all, the collection of saliva is easier than blood, its collection being non-invasive, stress-free and harmless to the patient. Saliva collection takes only a few minutes and provides both an adequate amount and volume of a specimen, even if repetition is necessary. In addition, saliva can be collected by individuals with limited training from almost all age groups, even those in which there are problems obtaining blood samples. It should also be highlighted that saliva is a cost-effective approach for the screening of large populations [5,6].

Saliva is produced by three pairs of major salivary glands (parotid, submandibular, and sublingual glands) and hundreds of small minor salivary glands. Saliva is composed mainly of water (99%), but also contains electrolytes, proteins, polypeptides, oligopeptides and small organic compounds, such as metabolites. Most of the organic compounds are produced in the salivary glands; however, some of them pass from the blood. These compounds migrate to saliva by three mechanisms, two intracellular (passive diffusion and active transport) and one extracellular (ultrafiltration) [6–10].

Saliva has the potential to become a first-line diagnostic sample of choice, especially for the monitoring of disease progression.

Abbreviations: DHEA, Dehydroepiandrosterone; EMA, European Medicines Agency; FDA, US Food and Drug Administration; IQR, Interquartile range; IS, Indoxyl sulphate; IS-d4, Indoxyl sulphate-D4; LC-MS/MS, Liquid chromatography coupled to mass spectrometry; pCS, *p*-Cresol sulphate; pCS-d7, *p*-Cresol sulphate-D7; QC, Quality control; RSD, Relative standard deviation; SD, Standard deviation; sIgA, Secretory immunoglobulin A

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Recently, experiments with the saliva from patients with renal failure were performed. It was observed that the concentration of serum creatinine (a renal function marker) was correlated with the concentration of renal markers in saliva, e.g., creatinine [6] and phosphate [11].

The aim of this study was to develop and validate a high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the analysis of pCS and IS in saliva. The influence of salivary flow and the type of swab used in the Salivette device were examined. The method was applied for the analysis of saliva samples of healthy volunteers of various ages. To the best of the authors' knowledge, this paper has for the first time described a repeatable and reproducible method for pCS and IS determination in saliva and the occurrence of measurable levels of uraemic toxins in the saliva of healthy individuals.

2. Materials and methods

2.1. Chemicals

Reference standard pCS and IS and internal standards *p*-cresol sulphate-d7 (pCS-d7) and indoxyl sulphate-d4 (IS-d4) were purchased from Toronto Research Chemicals (TRC, Canada). Solvents, HPLC gradient grade methanol, acetonitrile and formic acid 98% were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore water purification system (Milli-Q, Billerica, US).

2.2. Standard solutions, calibration standards and quality control samples

The stock solutions of the analysed compounds pCS, pCS-d7, IS and IS-d4 were prepared by accurate weighing of the appropriate amount of each compound and dissolving it in methanol to obtain a concentration of 1 mg mL^{-1}. The working standard solutions were prepared prior to use by dilution of the appropriate stock solutions with water to obtain the required concentrations. All stock solutions were stored at −26 °C. The calibration standards for pCS and IS were made at concentrations of 1.5–500 ng mL^{-1} and 1.5–200 ng mL^{-1}, respectively. The quality control (QC) samples were prepared at the following levels: pCS: 4.5, 250 and 500 ng mL^{-1}; IS: 2, 80 and 150 ng mL^{-1}. The calibration standards and QC samples were stored at −26 °C until required.

2.3. Sample preparation

The cotton swabs were centrifuged at 945*g* at 20 °C for 3 min to obtain saliva samples. The saliva samples were stored at −80 °C till the analysis. On the day of analysis the samples were deproteinised with acetonitrile (1 : 4, v/v), incubated at −20 °C for 20 min and centrifuged at 9300*g* at 4 °C for 10 min. The supernatant was analysed.

2.4. Chromatographic and mass spectrometric conditions

Instrumental analysis was performed using an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, US) equipped with a degasser, autosampler and binary pump, coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP 4000 (AB Sciex, Framingham, MA, US). The curtain gas, ion source gas 1, ion source gas 2 and collision gas (all high purity nitrogen) were set at 241 kPa, 413 kPa, 275 kPa and 'medium' instrument units (46–53 MPa), respectively. The ion spray voltage and source temperature were set at 4500 V and 600 °C, respectively. Chromatographic separation was achieved with a Kinetex C-18 column (100 mm, 4.6 mm, particle size 2.6 μm) supplied by Phenomenex (Torrance, CA, U.S.). The column was maintained at 40 °C at a flow rate of 0.5 mL min^{-1}. The mobile phases consisted of HPLC grade water with 0.1% formic acid as eluent A and methanol with 0.1% formic acid as eluent B. The gradient (B) was as follows: 0 min 10%; 0.5 min 10%; 4.5 min 95%; 8.5 min 95%. The injection volume was 10 μL. The target compounds were analysed in multiple reaction monitoring (MRM) mode, monitoring two transitions between the precursor ion and the most abundant fragment ions for each compound. The fragmentation patterns of the analytes are presented at Figs. A.1 and A.2. The transitions used for quantitation were m/z 186.9 > 106.9 and m/z 194.0 > 114.0 for pCS and pCS-d7, and m/z 211.9 > 79.8 and m/z 216.0 > 79.9 for IS and IS-d4, respectively. The compound parameters, viz. declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP), were −60, −38, −10, −5 V and −65, −46, −10, −1 V for IS and IS-d4, respectively: −65, −28, −10, −7 V and −60, −30, −10, −7 V for pCS and pCS-d7, respectively.

2.5. Application of the Salivette device

Assessment of the utility of the Salivette device in saliva collection for pCS and IS quantitation was performed. To estimate the possible retention of toxins on swabs, the cotton swabs (from Salivette) and synthetic swabs (from Salivette cortisol) were incubated in pooled saliva samples of known toxin concentration. The incubation was performed overnight at 4 °C. The swabs placed in test tubes were centrifuged at 945*g* at 20 °C for 3 min. The concentration of pCS and IS in the samples was compared with the concentration in saliva samples incubated without swabs.

2.6. Method validation

Material for validation experiments was the pooled saliva of healthy volunteers. To obtain the blank samples, the physiological level of pCS and IS was removed by incubation of the saliva with activated charcoal for 30 min at room temperature.

The validation was performed according to the EMA (European Medicines Agency) [12] and FDA (US Food and Drug Administration) [13] guidelines. Briefly, the linearity range was selected as 1.5–500 ng mL^{-1} for pCS and as 1.5–200 ng mL^{-1} for IS. Calibration curves were prepared in quadruplicate.

The accuracy and precision of the method were determined within run and between run using a lower limit of quantification (LLOQ) (Fig. 1A and B) and QC samples (4.5, 250 and 500 ng mL^{-1}) for pCS and QC samples (2, 80 and 150 ng mL^{-1}) for IS. Carry over was also studied.

Ion suppression or enhancement was initially studied based on the method of Matuszewski et al. [14] and allowed the quantitative assessment of the absolute and relative matrix effect by comparison of the analyte peak areas in the standard solutions and post-extraction spiked samples.

The stability of pCS and IS was confirmed in various conditions using QC samples. The tests included: freeze/thaw stability (three cycles) and short- (4 h at room temperature) and long-term (30 days at −20 °C) stability of analyte in the matrix. Stability in the autosampler, stock solution stability at different temperatures (−26 °C, 31 days; 4 °C, 7 days; room temperature, 1 day) and dilution integrity were also determined.

2.7. Influence of salivary flow

Fasting saliva was collected from 9 healthy people, aged between 20 to 62 (median = 24 years, interquartile range (IQR) = 29 years), according to the manufacturer's instructions. Collection was performed in the morning (between 7:00 and 10:00 a.m.) by a