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Development of a validated LC- MS/MS method for the quantification of 19 endogenous asthma/COPD potential urinary biomarkers

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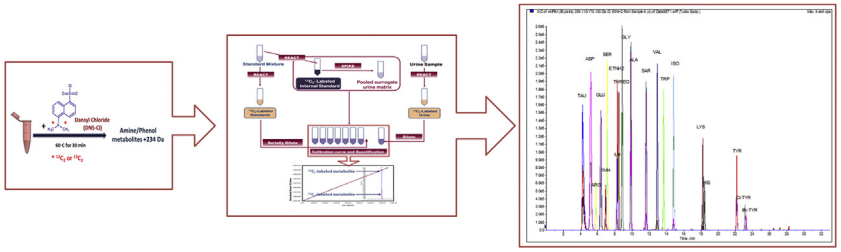
HIGHLIGHTS

- An HPLC-MS/MS method is developed for the absolute quantification of amino/phonel metabolites.
- Differential isotope labeling with dansyl chloride is applied.
- The method was fully validated according to FDA and EMA guidelines.
- Novel development and validation approaches were adopted to ensure the establishment of robust and reproducible method.
- Target urinary metabolites are quantified for asthma and COPD patients.

GRAPHICAL ABSTRACT



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ABSTRACT

Obstructive airways inflammatory diseases sometimes show overlapping symptoms that hinder their early and correct diagnosis. Current clinical tests are tedious and are of inadequate specificity in special population such as the elderly and children. Therefore, we are developing tandem mass spectrometric (MS/MS) methods for targeted analysis of urine biomarkers. Recently, proton-nuclear magnetic resonance (^{1}H-NMR) analysis proposed 50 urinary metabolites as potential diagnostic biomarkers among asthma and chronic obstructive pulmonary disease (COPD) patients. Metabolites are divided into 3 groups based on chemical nature. For group 1 (amines and phenols, 19 urinary metabolites), we developed and validated a high performance liquid chromatographic (HPLC)-MS/MS method using differential isotope labeling (DIL) with dansyl chloride. Method development included the optimization of the derivatization reaction, the MS/MS conditions, and the chromatographic separation. Linearity varied from 2 to 4800 ng/mL and the use of $^{13}C_2$-labeled derivatives allowed for the correction of matrix effects as well as the unambiguous confirmation of the identity of each metabolite in the presence of interfering isomers in urine. Despite the challenges associated with method validation, the method was fully validated as per the food and drug administration (FDA) and the European medicines agency (EMA) recommendations. Validation criteria included linearity, precision, accuracy, dilution integrity, selectivity, carryover, and stability. Challenges in selectivity experiments included the isotopic contributions of the analyte towards its internal standard (IS), that was addressed via the optimization of the IS concentration. In addition, incurred sample analysis was performed to ensure that results from patient samples are accurate and reliable. The method was robust and reproducible and is currently being applied in a cohort of asthma and COPD patient urine samples for biomarker discovery purposes.

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1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are a major cause of morbidity and they impose a huge economic burden with more than 600 million patients currently diagnosed with these illnesses [1]. Despite the distinct differences in the pathophysiology of asthma and COPD, their diagnosis using currently available diagnostic tools is difficult in a typical primary care setting [2–6]. It is common for some patients to experience an overlap in the clinical presentations of asthma and COPD or even have both as co-existing conditions [2,7,8]. This is particularly valid with asthmatic smokers, elderly with normally declined lung functions, and those with more severe asthma [2–4]. Correct diagnosis is crucial as each disease has different therapeutic strategies [4]. Accordingly, with the inaccessibility of accurate diagnostic tools in typical primary care settings, the diagnosis of asthma and COPD is too often based on history as described by the patient. Therefore, recent work has focused on novel approaches to diagnosis.

Metabolomics is the study of the end products of cellular metabolism in healthy and diseased states [9–12]. Applied metabolomics in medicine aims to link different biochemical pathways with disease diagnosis, severity, and therapeutic response [11,12]. It has demonstrated promising outcomes in biomarker discovery and in personalized medicine [11,13,14]. Among the various biological fluids, urine is ideal for metabolomic analysis. Its collection is non-invasive and it has richer metabolite content in comparison to blood, saliva, exhaled breath condensate or bronchoalveolar lavage [9,10]. Beyond lung disease, urine metabolomics has been mostly studied for improving the detection of various types of cancer [15,16], including that in the renal system [17,18]. Investigation of the urine metabolome has also included, but not limited to, the diagnosis of jaundice syndrome [19] and chronic heavy metal toxicity [20], the understanding of pathophysiology of depression [21] and the prediction of acute renal injury following cardiopulmonary bypass surgery in children [22].

Previous metabolomic studies on asthma or COPD subjects have been conducted in different biological fluids mostly in untargeted/semi-targeted approach [9,10]. For instance, a ^{1}H-NMR study of COPD blood samples showed lower levels of alanine, valine and isoleucine in comparison to healthy individuals [23]. Using the same platform, exhaled breath condensate (EBC) of COPD patients revealed significantly lower levels of valine and lysine, while the levels of serine and tyrosine were significantly higher in comparison to controls [24]. An increase in arginine levels and a decrease in glutamine, valine and isoleucine levels were also observed in the serum of COPD patients [25,26]. On the other hand, the serum of asthmatic patients, using ^{1}H-NMR, showed low levels of arginine along with valine and alanine, whereas; high histidine and glutamine levels were observed [27]. It can be concluded from these studies, that amino acids metabolism is an important pathway in the pathogenesis of asthma and COPD.

Pertaining to urine, comparisons of the metabolomic profiles of healthy participants with either asthmatic [28,29] or COPD subjects [30,31] have been reported. Despite providing useful metabolomic information, these 'disease/no disease' studies are less interesting in clinical practice. People know when they have chronic trouble breathing. The more important question is knowing the cause or type of chronic lung disease and the type of treatment it requires. Accordingly, metabolomics investigating the disease severity in asthma or COPD shows more promise for translational and personalized medicine in primary care settings [32–34]. However, previous reports either involved few specific classes of metabolites (e.g. volatile metabolites) [32,33] or resulted in the identity confirmation of very few metabolites [34].

To move to this important next stage, accurate targeted analysis is needed to clinically validate potentially therapeutic biomarkers. Contrary to the progress achieved in untargeted metabolomics, targeted quantification of endogenous metabolites is still at its infancy. Challenges in absolute quantification include the wide variations in metabolite concentration with diverse physicochemical properties [12]. In addition, neither endogenous metabolite-free matrices nor appropriate regulatory guidelines for the validation of bioanalytical methods for endogenous metabolites are available [35–37].

To the best of our knowledge, only 1 semi-targeted ^{1}H-NMR study investigated the differences in the urine metabolome between asthma and COPD patients [38]. Based on this investigation, a group of urine metabolites were suggested as potential diagnostic biomarkers differentiating asthma and COPD. Before these ^{1}H-NMR based metabolites can be used in a clinical lab, they require further validation. The suggested biomarkers were sub-divided into 3 groups based on their functional groups. Group 1 contains 19 metabolites that bear a primary amine, secondary amine or a phenolic group (Table 1). Group 2 contains 17 organic acids metabolites, whose identities and their validated quantification method will be described in a separate publication. Finally, group 3 contains 11 miscellaneous metabolites; including: quaternary ammonium compounds, sugars and nucleic acids as well as amino and organic acids that were not compatible with the developed methods for groups 1 and 2. Herein, we developed a fully validated LC-MS/MS method for the absolute quantification of a subset of 19 potential biomarker metabolites for the diagnosis of asthma and COPD. We adopted the deferential isotope labeling (DIL) approach [39] for the development of the quantification method. Amine and/or phenol functional groups contained within our metabolites can be derivatized with $^{12}C_2/^{13}C_2$-Dansyl chloride (DNS-Cl) as described by Guo et al. [39]. DIL has been mostly used for relative quantification purposes of the metabolome [39–42], however, despite the usefulness of the published semi-quantitative studies using DIL-DNS-Cl, we are required by regulatory bodies a full validation of the analytical method since clinical data will be obtained. The presented work discusses the usefulness of DIL strategy for absolute biomarker quantification and approaches to address the challenges typically faced in endogenous quantitative metabolomics.

2. Experimental

2.1. Materials and chemicals

All chemicals were purchased from Sigma Aldrich (Oakville, ON, CA) unless otherwise stated. 3-bromo tyrosine (98%) was purchased from abcam (Cambridge, UK) and sarcosine (99.4%) was purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA). Optima® LC-MS grade acetonitrile (ACN) and water was purchased from Fischer Scientific (ON, CA). The concentration of creatinine within subject samples was determined via Jaffe's colorimetric reaction using QuantiChrom™ creatinine assay kit (QC, CA) [43,44].

2.2. Synthesis of $^{12}C_2/^{13}C_2$-DNS-Cl

$^{13}C_2$-DNS-Cl bears 2- ^{13}C-methyl groups attached to the amino terminal within the naphthalene moiety of reagent. Structures of $^{12}C_2/^{13}C_2$ -DNS-Cl are compiled in Supplemental materials (Appendix I, Fig. 1). The $^{13}C_2$-labeled reagent was not available commercially. Accordingly, it was synthesized in house along with the non-isotopic form in a 2-step reaction procedure [39,45,46]. Synthesis protocol was modified from published methodologies in order to produce the highest yield of DNS-Cl (Supplemental materials, Appendix I, Fig. 1). The combination of the 2 step reaction is novel to the published methodology reported for the production of