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Simple, fast and sensitive LC–MS/MS analysis for the simultaneous quantification of nicotine and 10 of its major metabolites

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ABSTRACT

Urinary determination of nicotine metabolites provides an ideal tool for the quantitative assessment of the tobacco use-related nicotine dose, provided that the considered metabolites comprise a large share of the amount taken up. A method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed for the sensitive, fast and robust analysis of nicotine and 10 major nicotine metabolites ("Nic+10"), including cotinine, *trans*-3′-hydroxy-cotinine, nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, *trans*-3′-hydroxy-cotinine-*O*-glucuronide, nornicotine, norcotinine, nicotine-*N*′-oxide, cotinine-*N*′-oxide and 4-hydroxy-(3-pyridyl)-butanoic acid. Corresponding deuterated internal standards were spiked prior to a simple and straightforward solid phase extraction (SPE) procedure. Liquid chromatography was performed on a reversed phase C8 column and mass-specific detection was conducted in scheduled-MRM mode. The method was validated according to FDA Guidelines, showing excellent selectivity, precision, accuracy and robustness. The limits of quantification were in the range 0.2–2.3 ng/ml for all analytes. The novel method was applied to human urine samples derived from 25 smoking subjects. Quantitative results were correlated against a previously used LC–MS/MS method and compared to reports from the literature. The relative molar profile of nicotine and its 10 major metabolites was in good agreement with the literature. In addition, correlation amongst the two methods was excellent for almost all analytes, whereas the accordance between both methods was moderate for hydroxy-cotinine-*O*-glucuronide and norcotinine. These deviations, however, could be explained. The current method allows the simultaneous determination of nicotine and its 10 major metabolites (metabolite coverage about 95% of the absorbed dose) from a small sample volume and within a reasonable amount of time. Due to its wide dynamic range, high sensitivity and high throughput capabilities, this method could serve as a powerful tool for quantifying the nicotine dose of smokers, passive smokers as well as novel tobacco and nicotine product users in clinical and epidemiological studies.

1. Introduction

It is well known that tobacco consumption is one of the most critical public health problems, making tobacco use the leading cause of premature death in developed countries [1–3]. Tobacco alkaloids are the active principal components in all tobacco products. Among more than 20 different alkaloids found in tobacco, nicotine is the most abundant (98% of the total alkaloids) and accounts for widespread human use of tobacco products throughout the world, probably due to its pharmacological effects and possibly also its addictive potential [4–8]. Especially tobacco smoking is involved in a multitude of chronic diseases such as cancer (in particular lung cancer), cardiovascular diseases (CVD) and chronic obstructive pulmonary disease (COPD) [1]. Nicotine is rapidly and extensively metabolized by the liver in various compounds upon absorption in the human body [9,10]. Nicotine metabolism and relative amounts of the urinary metabolites are highlighted in Fig. 1. The predominant pathway during first pass metabolism is *C*-oxidation of nicotine to form cotinine. In humans about 70–80% of nicotine is converted to cotinine [11] which is subsequently hydroxylated, glucuronidated, oxidized and de-methylated to form various cotinine-derived metabolites (Fig. 1) [9]. *N*-Oxidation is also a primary route of nicotine metabolism, although only 4–7% of the nicotine amount absorbed by smokers is being transformed via this direction [9]. In addition to the oxidation of the pyrrolidine

Abbreviations: COPD, chronic obstructive pulmonary disease; FDA, Food and Drug Administration; Nic, nicotine; Cot, cotinine; OH-Cot, *trans*-3′-hydroxycotinine; Nic-Gluc, nicotine-*N*-glucuronide; Cot-Gluc, cotinine-*N*-glucuronide; OH-Cot-Gluc, *trans*-3′-hydroxy-cotinine-*O*-glucuronide; NorNic, nornicotine; NorCot, norcotinine; NNO, nicotine-*N*′-oxide; CNO, cotinine-*N*′-oxide; HyPyBut, 4-hydroxy-(3-pyridyl)-butanoic acid; UGT, UDP-glyucuronosyltransferase; FMO, flavin containing monooxygenase.

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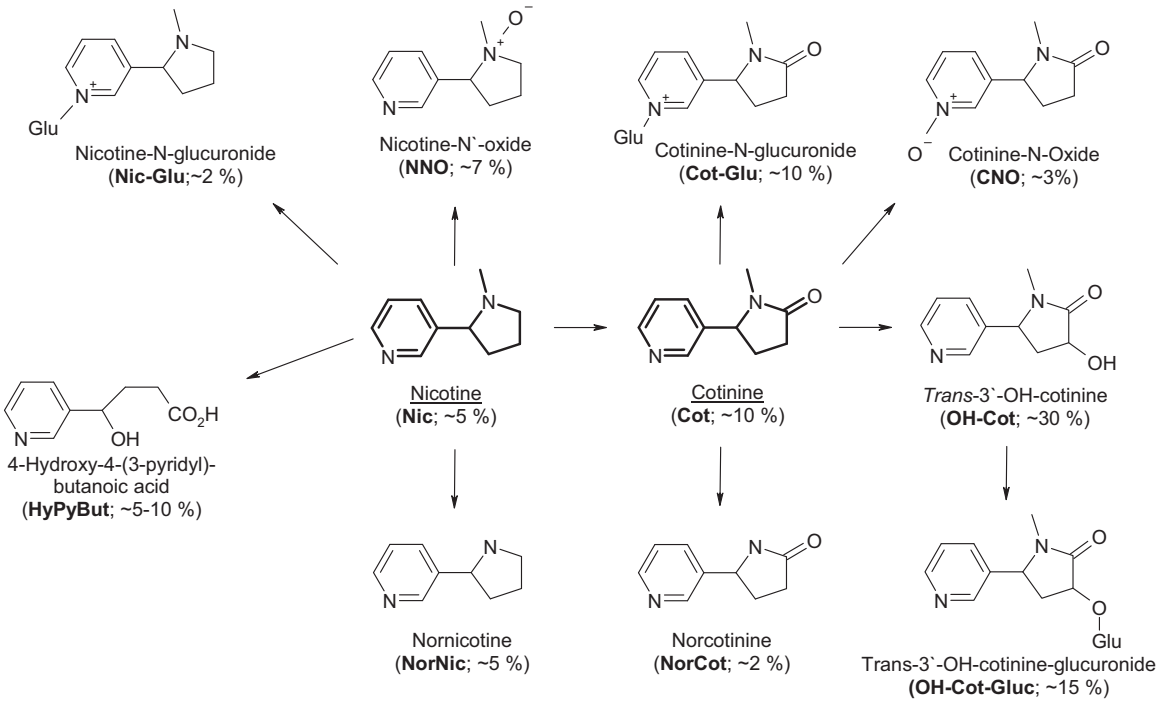

Fig. 1. Metabolism of nicotine to its major urinary metabolites. Abbreviations and relative amounts of metabolites in human urine are given in parenthesis, estimated from own and published data [9].

ring, nicotine is also de-methylated and glucuronidated to form the minor metabolites Nor-Nicotine and Nicotine-*N*-glucuronide, respectively. These metabolites account only for 5% respectively 2% of the nicotine dose absorbed [11]. About 10–15% of nicotine and metabolites is excreted as 4-oxo-4-(3-pyridyl)-butanoic acid and 4-hydroxy-4-(3-pyridyl)-butanoic acid in smokers urine (Fig. 1) [12]. For the first time in the year 2000, Hecht and colleagues have shown [12] that these metabolites arise directly from a nicotine transformation and not as initially thought from cotinine [13].

The determination of cotinine in blood, saliva and urine is well established and has been most frequently used as a biomarker of nicotine and tobacco smoke exposure [3,10]. Its suitability and limitations of this purpose has been extensively reviewed [14–18].

If a high percentage of the metabolites excreted in urine is covered, the determination of nicotine and its major metabolites provides a useful tool for estimating the total nicotine dose obtained by the various forms of tobacco use, which is difficult to quantify by other methods In contrast to measuring only single or a few metabolites, the high coverage rate of nicotine metabolites would also have the advantage that factors which affect the metabolism such as genetics, gender, enzymatic induction, inference with other chemicals would be of only minor importance. Various combinations of urinary nicotine metabolites have been applied measuring the nicotine uptake by smoking and other tobacco uses (for review, see [18]). Most frequently, nicotine and its major metabolites cotinine, *trans*-3′-hydroxy-cotinine, nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, *trans*-3′-hydroxy-cotinine-*O*-glucuronide, nornicotine, norcotinine, nicotine-*N*′-oxide and cotinine-*N*′-oxide have been determined in urine for this purpose. The chemical structures as well as approximate percentages of the nicotine uptake dose excreted in urine are shown in Fig. 1. The nicotine dose based on these 10 urinary metabolites (also called "Nic+9") make up approximately 90% of the amount taken up. In three studies, "Nic+8" was determined by omitting nornicotine [19–21]. In other studies, the 10 mentioned metabolites without norcotinine [22] and cotinine-*N*′-oxide [23] was determined. The proportions of the metabolites were found to be similar in the various studies [18] (Fig. 1). "Nic+8" (nornicotine omitted) was also applied for nicotine dose measurements in two smoking behavior studies, however, without providing percentage for the single urinary metabolites [24,25].

Nicotine equivalents comprising "Nic+5" (nicotine, cotinine, OH-Cot and their respective glucuronides) represent approximately up to 80% of the nicotine [26]. [18]. "Nic+5" has been frequently applied as biomarker for the smoking- and tobacco use-related exposure to nicotine in recent years [27–34].

"Nic+5" equivalents in urine are determined by 'indirect' or 'direct' methods. Indirect methods comprise two analytical runs: (i) Determination of free bases (aglycons) in untreated urine and (ii) determination of the aglycons after enzymatic hydrolysis of the urine with ß-glucuronidase. The first determination provides values for the free bases (nicotine, cotinine, *trans*-3′-hydroxycotinine). The second determination provides values for total bases (free + conjugated metabolite). The difference between the second and first determination represents the amount of the conjugated metabolite. Advantages of the indirect approach include the application of both gas chromatography (GC) and liquid chromatography (LC) with various detectors (NPD, MS, MS/MS), additionally, no unlabeled and labeled glucuronides are required as reference materials or internal standards, respectively. The disadvantage of the indirect approach is the fact that two separate analytical runs have to be performed and that the analytical variation for the glucuronides is, therefore, increased. This approach was used by various groups [22,26,35–37]. Direct methods [21,33,38] allow the determination of nicotine equivalents in urine in one run and do not require the enzymatic hydrolysis step. Disadvantages of this approach are requirement of relatively expensive analytical instrumentation as well as of unlabeled and labeled standards for the glucuronides.

Of the three principal methodological approaches for the determination of nicotine metabolites in urine, namely LC, GC, immunological methods (for review, see [39]), LC–MS/MS is the most promising approach, because it allows the determination of the whole range of analytes, despite their largely deviating