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Short Communication

Anabasine analysis in human plasma using liquid chromatography coupled to tandem mass spectrometry to Verify tobacco use: empirical challenges and limitations

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ABSTRACT

Anabasine is an alkaloid frequently quantified by LC–MS/MS to differentiate tobacco use from nicotine replacement therapy. While urine provides reliable measurement, plasma remains a poorly validated and analytically challenging matrix. This study assessed widely used sample preparation strategies for anabasine determination in human plasma. Across all methods, recovery in undiluted plasma was highly variable and largely outside acceptable analytical ranges, with marked ion suppression and poor reproducibility. Matrix dilution increased apparent signal but substantially worsened variability. Experiments in albumin-enriched saline excluded protein binding as the main determinant of analyte loss, indicating broader plasma-related matrix effects. In human plasma, including time-course sampling during and after smoking, anabasine remained consistently below quantifiable levels. None of the tested workflows met the robustness criteria required for quantitative LC–MS/MS analysis, indicating that current preparation approaches do not enable reliable anabasine measurement in plasma, a critical limitation for studies using anabasine as a biomarker of tobacco exposure.

1. Introduction

Tobacco-specific alkaloids are routinely measured by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) to assess tobacco exposure, particularly in contexts where nicotine replacement products are used [1]. Nicotine substitutes contain purified nicotine and lack minor tobacco alkaloids naturally present in tobacco leaves. Therefore, anabasine is widely used to distinguish nicotine substitution from continued tobacco use because of its structural similarity to nicotine (Fig. S1) [2,3]. Anabasine is therefore commonly integrated into multi-analyte LC–MS/MS panels alongside nicotine, cotinine, and related metabolites, and is quantified under the same

Abbreviations: ACN-PP, Acetonitrile Protein Precipitation; ACN-PP-DIL, Acetonitrile Protein Precipitation with Dilution; BSA, Bovine Serum Albumin; CE, Collision Energy; EDTA, Ethylenediaminetetraacetic Acid; ENZ-HYD, Enzymatic Hydrolysis; ESI, Electrospray Ionization; GC, Gas Chromatography; HLB, Hydrophilic-Lipophilic Balance; ISTD, Internal Standard; LC-MS/MS, Liquid Chromatography–Tandem Mass Spectrometry; LOD, Limit of Detection; LOQ, Limit of Quantification; MRM, Multiple Reaction Monitoring; NRT, Nicotine Replacement Therapy; PBS, Phosphate Buffered Saline; RPD, Relative Percent Difference; RT, Retention Time; SAL-DIL, Plasma Dilution with Salin; SHCS, Swiss HIV Cohort Study; SPE, Solid-Phase Extraction.

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chromatographic and mass-spectrometric conditions [4,5]. Urine is considered the most reliable matrix for anabasine determination [6,7], whereas plasma has received limited analytical characterization, and the few available studies report substantial challenges, including strong matrix effects, inconsistent recovery, and high variability [4,8]. A recent proficiency-testing survey also highlighted poor comparability among laboratories for plasma measurements of minor tobacco alkaloids including anabasine [9]. Despite these issues, plasma is occasionally the only biological sample available in clinical or research settings, and several studies continue to attempt anabasine measurement in this matrix, reinforcing the need for a systematic analytical evaluation. To address these uncertainties and reflect current laboratory practice, this study evaluates whether anabasine can be reliably quantified in plasma using the same LC–MS/MS workflow applied to nicotine and its metabolites. Widely used preparation methods - protein precipitation, solid-phase extraction, enzymatic hydrolysis, and matrix dilution - were assessed for recovery, matrix effects, precision, and calibration linearity.

2. Materials and methods

2.1. Plasma samples and reagents

Anonymized plasma samples were obtained from two clinical studies and stored at −20 °C or − 80 °C. The first dataset comprised Swiss HIV Cohort Study (SHCS) samples (n = 16; ten smokers, six non-smokers). The second consisted of nicotine toxicokinetics samples (n = 16 smokers) collected at defined time points before, during, and after a controlled smoking session (baseline; 1- and 3- min during smoking; end of smoking; 5-, 12-, 24- and 30-min post-smoking). Anabasine, nicotine-d_4 (internal standard, ISTD), formic acid, methanol, ammonium hydroxide, and ethyl acetate were purchased from Sigma-Aldrich. Acetonitrile was obtained from PanReac AppliChem, and nanopure water from an ariumPro ultrapure system (Sartorius). Phosphate-buffered saline (PBS) was prepared in-house (8 g/L NaCl, 2.72 g/L $Na_2HPO_4{\cdot}7H_2O$, 0.245 g/L KH_2PO_4, 0.2 g/L KCl), and the enzymatic mixture used for hydrolysis experiments was prepared as described in Section S1.

2.2. Sample preparation and experimental approach

All blood samples were collected in EDTA tubes. Plasma was isolated by centrifugation (SHCS: 3000 rpm, 8 min, 25 °C; toxicokinetics study: 1300 rpm, 12 min, 4 °C). Five preparation workflows were evaluated: acetonitrile protein precipitation (ACN-PP), solid-phase extraction with HLB cartridges (SPE), protein precipitation followed by dilution (ACN-PP-DIL), enzymatic hydrolysis followed by liquid–liquid extraction (ENZ-HYD) and serial matrix dilution (DIL). Their operational steps and analytical rationale are summarised in Table S1. Recovery and matrix effects were then assessed in plasma from confirmed non-smokers spiked at 5.0 and 20.0 ng/mL using ACN-PP, SPE, ACN-PP-DIL, and ENZ-HYD. Direct calibration curves (0.01–20 ng/mL) were applied for all methods except SPE, which required an extracted curve.

2.3. Chemical analysis

Chemical analyses were performed using an LCMS-8060NX system (Shimadzu) with a Shim-pack GIST C18 metal-free column. Separation used a water–methanol gradient with 0.05% formic acid, and detection was carried out in ESI+ multiple reaction monitoring (MRM) mode. Full chromatographic and ion-source parameters are reported in Table S2. LOD and LOQ were based on signal-to-noise ratios of 3 and 10. Analytical performance was evaluated using linearity ($r^2 > 0.995$), recovery (70–130%) and precision expressed as relative percent difference (RPD < 20%) [10,11].

2.4. Control matrices and preparation of artificial samples

To assess matrix-related effects on anabasine recovery, two artificial matrices were prepared: PBS and PBS supplemented with 5% w/v bovine serum albumin (BSA). Samples were spiked with anabasine (5.0 ng/mL) and nicotine-d_4 (10.0 ng/mL). All preparations were processed using the ACN-PP protocol.

2.5. Ethical approval

The use of biological samples from both studies was approved by the ethics committee (Commission cantonale d'éthique de la recherche sur l'être humain vaudoise: BASEC 2023–02080 for SHCS and BASEC 2018–00332 for toxicokinetic study). All participants provided written informed consent.

3. Results

3.1. Detection and mass spectrometry parameters

Anabasine was quantified by LC–MS/MS in MRM mode (Table 1), with chromatographic and ion-source conditions detailed in Table S2. Deuterated nicotine was used as ISTD to ensure alignment with tobacco-alkaloid workflows previously published [4,6,12]. Representative chromatograms are shown in Fig. S2.

3.2. Linearity and analytical recovery by spiking

The direct calibration curve showed excellent linearity (Table 2, Fig. S3), yielding an LOQ of 0.10 ng/mL and an LOD of 0.03 ng/mL. In contrast, the extracted calibration curve generated using the SPE workflow displayed poor linearity (Table 2), indicating that SPE did not support reliable quantitative calibration under the tested conditions. Recovery was then evaluated in plasma from confirmed non-smokers (Table 2). The four preparation methods (ACN-PP, SPE, ACN-PP-DIL and ENZ-HYD), applied as outlined in Table S1 and within the workflow of Table S3, produced the recovery values reported in Table 2. A representative chromatogram for plasma spiked at 5.0 ng/mL is shown in Fig. S4. ENZ-HYD yielded low but reproducible recoveries, whereas ACN-PP showed marked signal instability with both under- and over-quantification. Among all protocols, ACN-PP-DIL produced recoveries closest to the expected quantitative range, despite substantial variability, and was therefore selected for subsequent analyses.

3.3. Analytical recovery during matrix dilution

Matrix dilution was evaluated as an additional strategy to reduce matrix-related interferences. Plasma samples were spiked with anabasine at 20.0 ng/mL and diluted in nanopure water prior to analysis (Table 3). Overall, recovery increased with higher dilution, with the highest dilution factor yielding the best apparent recovery. However, all dilution levels were characterized by substantial variability, and lower dilution factors showed both reduced recovery and limited reproducibility. These findings indicate that matrix dilution can improve signal

Table 1
LC-MS/MS parameters for the detection of anabasine and the internal standard nicotine-d4.

Compound Name	RT	Target Ion (m/z)	CE Target Ion	Reference Ion (m/z)	CE Reference Ion
Anabasine	5.37 min	163.10 > 118.15	−24.0 eV	163.10 > 146.20	−15.0 eV
Nicotine-d4	4.33 min	167.00 > 134.15	−23.0 eV	167.10 > 121.25	−26.0 eV

RT: retention time; CE: collision energy.

Table 2
Anabasine recovery (%) by doping stage across sample preparation methods. Each recovery value represents the mean and standard deviation of three independent measurements.

Method	Linearity (R^2)	Rec%[1] Plasma [Anab][2]: 5 ng/mL	Rec%[1] Plasma [Anab][2]: 20 ng/mL
ACN-PP	0.998	8% ± 247%	402% ± 110%
SPE	0.178	ND	ND
ACN-PP-DIL	0.998	−580 ± 445%	184% ± 79%
ENZ-HYD	0.998	13% ± 2%	16% ± 1%

ACN-PP: Acetonitrile Protein Precipitation; ACN-PP-DIL: Acetonitrile Protein Precipitation with a dilution step; ENZ-HYD: Enzymatic Hydrolysis; SPE: Solid-Phase Extraction; ND: not determined.

[1] Rec% indicates the percentage of analyte recovered after preparation and analysis, each value represents the mean and standard deviation of three independent measurements.

[2] [Anab] refers to the nominal concentration of anabasine used for spiking.

Table 3
Anabasine recovery (%) at increasing plasma dilution levels using the DIL method. Each value represents the mean and standard deviation of four independent measurements.

Dilutions[1]	Recovery[2]
1:500	121% ± 64%
1:100	63% ± 43%
1:50	49% ± 45%
1:25	19% ± 52%

[1] Dilution ratio of plasma to nanopure water (plasma: nanopure water).

[2] Mean and standard deviation calculated from three independent triplicates.

recovery under highly diluted conditions but does not resolve the variability associated with plasma-based quantification.

3.4. Evaluation of albumin-related effects on anabasine recovery

To assess whether plasma protein content contributed to the low recoveries observed in plasma, recovery experiments were performed in 5% BSA matrices within the workflow described in Table S3. Samples were spiked with anabasine at 5.0 ng/mL and processed using the ACN-PP-DIL protocol. Recovery in the albumin-enriched matrix was high and consistent (88% ± 17%), indicating that albumin alone does not substantially limit anabasine availability and is unlikely to explain the poor recoveries observed in native plasma.

3.5. Analysis of human plasma samples

A total of 16 human plasma samples were analysed, originating from individuals classified as active smokers or non-smokers. All samples were processed using the ACN-PP-DIL protocol. Among the analysed samples, only one showed a quantifiable concentration of anabasine, measured at 0.80 ng/mL (Table S4). This positive measurement corresponded to a sample classified as non-smoker. A representative chromatogram of this positive sample is shown in Fig. S5.

3.6. Anabasine concentration during and after active smoking

A toxicokinetics study was conducted in volunteer smokers to assess the short-term detectability of anabasine in plasma. Quantitative results and corresponding recovery values from spiked controls are reported in Table S5. At all sampling points, anabasine concentrations remained below the LOQ. Recovery values from spiked controls at 20 ng/mL processed in parallel showed pronounced variability across the time series, indicating that inconsistent extraction performance -rather than biological kinetics -accounts for the absence of detectable anabasine in plasma.

3.7. Study strenghts and limitations

This study has several strengths, including the use of state-of-the-art LC–MS/MS instrumentation and widely adopted extraction workflows for plasma analysis [13–15]. The analytical strategy was deliberately based on a classical LC–MS/MS configuration using conventional reversed-phase chromatography (C18-type columns), to ensure broad applicability and relevance for laboratories performing routine and large-scale analyses [8,14,16]. Fresh and frozen plasma samples were included to reflect real pre-analytical conditions. Limitations include the absence of paired urine samples and the use of nicotine-d_4 instead of a deuterated anabasine analogue. Although this choice may introduce minor differences in ionization efficiency and thus affect linearity and analytical recovery, poor sensitivity remains a major limitation and is not expected to be substantially improved by using of a deuterated anabasine as ISTD. Overall, the high chromatographic quality obtained supports the suitability of nicotine-d_4 as ISTD in this context. Further, only one SPE-based preparation protocol was used, focusing exclusively on the Oasis HLB cartridge. This cartridge was selected because it is among the most widely used SPE sorbents and offers a broad retention spectrum for acidic, neutral, and basic compounds, as well as for analytes with high hydrophilicity. Moreover, previous studies have shown that the Oasis HLB cartridge provides higher recoveries in urine compared with other SPE columns [5,6]. However, other SPE cartridges based on strong cation exchange and mixed-mode reversed-phase sorbents could also be considered.

4. Discussion

This study provides the first systematic evaluation of anabasine recovery from human plasma across widely used LC–MS/MS preparation workflows. All methods yielded recovery values outside acceptable analytical ranges and showed poor repeatability, confirming the strong matrix-dependent limitations previously suggested in the literature [8,9]. Matrix dilution improved apparent signal but did not resolve variability, and experiments with BSA showed high recovery, indicating that albumin is not responsible for analyte loss. These findings point instead to broader plasma-related interferences known to affect the extraction of small alkaloids [17]. Real plasma samples, including specimens collected during and after active smoking, consistently showed anabasine concentrations below the LOQ, in line with the limited detectability reported in previous plasma studies [4,12]. Collectively, the data demonstrate that none of the tested workflows provides reliable quantitative performance for anabasine in plasma, reinforcing evidence that urine is the biologically and analytically suitable matrix for this biomarker [2,16,18].

5. Conclusions

This study demonstrates that human plasma is a highly challenging matrix for the quantitative determination of anabasine using commonly available sample preparation approaches coupled to standard LC–MS/MS analysis. Across all tested protocols, recovery and reproducibility remained consistently inadequate due to persistent matrix-related interferences. While these results indicate that straightforward quantification is not achievable with routine analytical workflows, they also highlight the need for further method development, including the exploration of more sophisticated approaches such as alternative chromatographic selectivities, derivatization strategies, GC-based methods leveraging the volatility of anabasine, optimized liquid–liquid extraction schemes (e.g., acid–base partitioning), and improved control of analyte volatility to enhance sensitivity and mitigate matrix effects.

CRediT authorship contribution statement

Christof Manuel Schönenberger: Writing – original draft, Funding acquisition, Conceptualization. **Aurelie Berthet:** Writing – review & editing, Conceptualization. **Matthias Briel:** Writing – review & editing, Supervision, Funding acquisition. **Alain Amstutz:** Writing – review & editing, Supervision, Funding acquisition. **Matthias Cavassini:** Writing – review & editing. **Loïc Sartori:** Writing – review & editing, Investigation. **Camille Rime:** Writing – review & editing, Investigation. **Davide Staedler:** Writing – original draft, Validation, Supervision, Resources, Project administration, Formal analysis, Conceptualization. **Fiorella Lucarini:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2026.124942.

Data availability

Data will be made available on request.

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