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Simultaneous quantification of purine and pyrimidine bases, nucleosides and their degradation products in bovine blood plasma by high performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Improved nitrogen utilization in cattle is important in order to secure a sustainable cattle production. As purines and pyrimidines (PP) constitute an appreciable part of rumen nitrogen, an improved understanding of the absorption and intermediary metabolism of PP is essential. The present work describes the development and validation of a sensitive and specific method for simultaneous determination of 20 purines (adenine, guanine, guanosine, inosine, 2′-deoxyguanosine, 2′-deoxyinosine, xanthine, hypoxanthine), pyrimidines (cytosine, thymine, uracil, cytidine, uridine, thymidine, 2′-deoxyuridine), and their degradation products (uric acid, allantoin, β-alanine, β-ureidopropionic acid, β-aminoisobutyric acid) in blood plasma of dairy cows. The high performance liquid chromatography-based technique coupled to electrospray ionization tandem mass spectrometry (LC–MS/MS) was combined with individual matrix-matched calibration standards and stable isotopically labelled reference compounds. The quantitative analysis was preceded by a novel pre-treatment procedure consisting of ethanol precipitation, filtration, evaporation and reconstitution. Parameters for separation and detection during the LC–MS/MS analysis were investigated. It was confirmed that using a log-calibration model rather than a linear calibration model resulted in lower CV% and a lack of fit test demonstrated a satisfying linear regression. The method covers concentration ranges for each metabolite according to that in actual samples, e.g. guanine: 0.10–5.0 μmol/L, and allantoin: 120–500 μmol/L. The CV% for the chosen quantification ranges were below 25%. The method has good repeatability (CV% ≤ 25%) and intermediate precision (CV% ≤ 25%) and excellent recoveries (91–107%). All metabolites demonstrated good long-term stability and good stability within-runs (CV% ≤ 10%). Different degrees of absolute matrix effects were observed in plasma, urine and milk. The determination of relative matrix effects revealed that the method was suitable for almost all examined PP metabolites in plasma drawn from an artery and the portal hepatic, hepatic and gastrosplenic veins and, with a few exceptions, also for other species such as chicken, pig, mink, human and rat.

1. Introduction

The global efficiency of nitrogen in animal production is only slightly over 10%, with the result that 102 Tg (10^{12} gram) nitrogen is excreted annually (1998 figures) by domesticated animals globally [1]. The nitrogen efficiency in dairy cows is generally low [2], and not only the environment, but also the productive efficiency, would benefit from an optimization of diet and metabolism to improve nitrogen efficiency and utilization [1,3,4]. Most research hitherto has focused on refining protein and amino acid utilization, but this has only led to minor improvements in efficiency [4–6]. A better understanding of the quantitative absorption and intermediary metabolism of other nitrogenous products such as the purines and pyrimidines (PP), the building blocks of nucleic acids and main constituents of DNA/RNA, could uncover new ways of improving

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dairy cow nitrogen use-efficiency and propose new feeding strategies [7,8]. So far, the possible significance of microbial PP in the nutritional physiology of ruminants has not been investigated, regardless of the fact that they correspond to more than 20% of the total microbial nitrogen supply [7–9]. Little is known about the quantitative aspects of PP metabolism. What is known, however, is that the purines go through an effective multistep degradation to uric acid and allantoin, and the pyrimidines are similarly degraded to β-alanine, before excretion [8,10].

Quantitative analysis of PP in dairy cattle research has almost solely focused on purines in urine, as excretion of purine derivatives can be used as an indirect measure of rumen microbial synthesis [11–14]. Most published methods have thus been developed for purine metabolites in urine. Only recently, Boudra et al. published a method able to quantify the pyrimidine degradation products (DP) β-alanine and β-aminoisobutyric acid as well [14].

Different analytical separation methods have been used for determining PP in biological matrices of which the majority has applied high performance liquid chromatography (HPLC) [15–17] or capillary electrophoresis chromatography [17–20]. When high separation selectivity and sensitivity were essential, electrokinetic techniques [16] or ultra high performance liquid chromatography [21] have been used. Concerning detection, spectrometric, electrochemical or mass spectrophotometric detection methods have been used, with ultra violet detection coupled to HPLC being the most common one [15–17]. HPLC coupled with tandem spectrometric detection (LC–MS/MS) is currently considered the method of choice for quantitative analysis of compounds in biological matrices [22] and LC–MS/MS has been shown to be capable of quantifying PP and their derivatives accurately in urine.

For this study, we wanted to develop and validate an LC–MS/MS method for quantification of a range of PP and their derivatives in cow blood plasma. Into this procedure, we wanted to incorporate matrix-matched calibration standards as well as stable isotopically labelled reference compounds (SIL). As no appropriate pre-treatment procedure was identified in the literature, we also wanted to develop a good, stable, simple, component-specific, and repeatable pre-treatment protocol for the plasma samples.

Several sets of plasma samples from experiments that attempted to manipulate urea-recycling and increase nitrogen utilization using multicatheterized Danish Holstein cows were employed in the development of this method [23] because these were representative of the types of samples that this method is likely to be used for in the future.

2. Materials and methods

2.1. Chemicals, reagents and materials

Water quality was at all times secured by treatment on a Millipore Synergy® UV water treatment system from Millipore A.S. (Molsheim, France). Methanol (MeOH) from Poch S.A. (Gliwice, Poland) and ethanol (EtOH 99.9% vol.) from Kemetyl A/S (Køge, Denmark) were of HPLC grade. Formic acid (98–100%) (HCOOH), acetic acid (100%) (CH_3COOH), and ammonium solution (25%) (NH_4OH) from Merck (Darmstadt, Germany) were of analytical reagent grade. Sodium hydroxide (NaOH), also from Merck, was prepared in a 0.01 M aqueous solution. Tricholoroacetic acid (≥99.0%) from Sigma-Aldrich (Brøndby, Denmark) was prepared in a 12% (v/v) aqueous solution (TCA) daily. Contamination between samples was minimized by the use of disposable materials (vials, bottles, etc.) where practicable, or through the use of lab equipment that was cleaned without the use of detergents.

2.2. Standards

The following compound standards (bases (BS), nucleosides (NS), DP) were obtained from Sigma-Aldrich (Brøndby, Denmark): adenine, guanine, cytosine, thymine, uracil, adenosine, guanosine, cytidine, uridine, inosine, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, thymidine, 2′-deoxyuridine, 2′-deoxyinosine, xanthine, hypoxanthine, uric acid, allantoin, β-alanine, β-ureidopropionic acid and β-aminoisobutyric acid. β-ureidoisobutyric acid, one important intermediate pyrimidine derivate metabolite, was not commercially available and could not be included. No traces of either adenosine or 2′-deoxyadenosine were identified during method development in plasma or urine samples. 2′-deoxycytidine was present in trace amounts but even after extensive optimization the sensitivity remained too low for quantification. These three components were therefore not pursued further. The chemical structures of the targeted metabolites are shown in Table 1.

Stable isotopically labelled reference compounds used as internal standards were purchased from Cambridge Isotope Laboratories (Andover, USA). These were: adenine (8-^{13}C), guanine (8-^{13}C;7,9-^{15}N2), thymine (^{15}N2), uracil (U-^{13}C4;U-^{15}N2), guanosine (U-^{13}C10;U-^{15}N5), inosine (U-^{15}N4), cytidine (U-^{13}C9;U-^{15}N3), uridine (U-^{13}C9;U-^{15}N2), 2′-deoxyguanosine (U-^{15}N5), thymidine (U-^{15}N2), xanthine (1,3-^{15}N2), hypoxanthine (^{15}N4), uric acid (1,3-^{15}N2), and β-alanine (U-^{13}C3;^{15}N). Cytosine (2,4-^{13}C2;^{15}N3) was purchased from Sigma-Aldrich (Brøndby, Denmark). All were ^{13}C and/or ^{15}N labelled with purities of at least 95% (95–99%). Unfortunately, exact SIL were not available for all metabolites studied; a suitable SIL was consequently selected on its similarity to the corresponding metabolite in terms of structure, retention time, fragmentation pattern and group. Individual stock solutions of all compound standards and SIL were prepared and kept at −80 °C. Bases and purine DP were diluted in water and NS and pyrimidine DP were diluted in 0.01 M NaOH solution. Two stock concentrations of 500 and 5000 μmol/L were made for each compound standard. The exception was for uric acid and allantoin, where the stock concentration was 500/2000 and 500/40,000 μmol/L, respectively. For SIL only the low concentration stock was prepared. All stocks were filtered through 0.45 μm PALL GHP Membrane syringe filters purchased from VWR (Herlev, Denmark) and kept at −20 °C in dark vials. Appropriate dilutions of these solutions were made in water to produce standard mixtures and SIL mixtures for external calibration and quantification.

2.3. Samples

A number of 5 mL aliquots of heparinized plasma to be used for external calibration and quality control were prepared from 2 L of venous blood [23] drawn from a Danish Holstein dairy cow fed a traditional total mixed ration. Experimental plasma samples were obtained from a feeding experiment [24] with multicatheterized dairy cows [25,26]. This set of samples was drawn from four blood vessels simultaneously, representing blood from an artery and the portal hepatic, hepatic and gastrosplenic veins. Additional test plasma samples were obtained on site for relative matrix effect evaluations. These samples were from five other species (chicken, pig, mink, human, and rat) for between species comparisons, four multicatheterized cows (jugular vein) for intraspecies comparisons, and bovine urine and milk samples for matrix effect evaluations.

2.4. Pre-treatment

Before pre-treatment, plasma samples for quantification of uric acid and uracil were diluted twenty-fold (5%, v/v) and four-fold