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Simultaneous determination of creatinine and pseudouridine concentrations in bovine plasma by reversed-phase liquid chromatography with photodiode array detection

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Abstract

A simple, rapid method for the simultaneous determination of creatinine and pseudouridine in bovine plasma is described. Plasma was de-proteinised, concentrated, and chromatographed for 15 min on a C_{18} column. Analytes were detected at an optimum wavelength (262 nm) and the internal standard (cimetidine) was detected at 220 nm. The pH of analysis was between 6.5 and 7 where both analytes exist in single chemical forms giving maximum accuracy. Recoveries of both analytes were above 96%. Lowest detectable amounts of creatinine and pseudouridine were 0.28 nmol and 9.0 pmol, and the typical levels detected (±SD) were 60 (±2.8) and 2.3 (±0.10) μmol/L, respectively.

Keywords: Creatinine; Pseudouridine; Cimetidine

1. Introduction

Creatinine and pseudouridine are used as metabolic indicators of skeletal muscle mass [1] and protein synthesis [2], respectively. Creatinine is used as an indicator of skeletal muscle mass because it is a by-product of the creatine kinase reaction, 98% of which occurs in skeletal muscle during short-term energy repletion. Creatinine forms spontaneously from the cyclization of creatine that is produced from this reaction following the dephosphorylation of creatine phosphate. Pseudouridine is released during the degradation of transfer and ribosomal ribonucleic acids and is not salvaged by the body once released from the tissues in ruminants [3]. Consequently, it has been used as an indicator of protein synthesis, assuming that increased protein synthesis requires increased RNA synthesis and degradation [2].

The concentration of pseudouridine in urine increases with body weight; therefore, it is expressed in ratio with creatinine in order to correct for tissue mass. This ratio has been shown to reflect changes in the protein accretion status of ruminants during growth, pregnancy or lactation [4,5]. Assessment of the amount of pseudouridine and creatinine excreted over 24 h by ruminants requires that the animals be

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housed for many days in metabolic crates that restrict their movement and compromise the comfort of the experimental animals. Simultaneous measurement of these metabolites in a blood sample, however, would be valuable because obtaining a blood sample from a ruminant animal is often less stressful than housing in a metabolic crate.

Review of the recent literature revealed that the method of choice for the measurement of creatinine has been RPLC. Recent developments of methodology were available for the measurement of creatinine in human serum and urine [6], for the measurement of pseudouridine in human urine [7,8], for the measurement of both creatinine and pseudouridine in bovine urine [9], and for the measurement of pseudouridine in human serum [10]. These methods exhibited either lengthy run times and complicated HPLC procedures, absence of internal standard in quantitation, analysis at a pH where more than one chemical form of creatinine exist in samples, or lack of sensitivity for pseudouridine relative to creatinine in samples. Only a few methods were found that reported the determination of pseudouridine in human serum, and no methods were found that determined creatinine and pseudouridine simultaneously in bovine plasma. Even a recently reported method of measuring pseudouridine in human serum [10] involved a lengthy and complicated extraction procedure, and it has not been validated for bovine serum.

We present here a simultaneous method of determination for creatinine and pseudouridine that is simple yet robust. Accuracy and precision are enhanced with this method through the use of optimum wavelengths for each analyte and the internal standard with a photodiode array (PDA) detector. The identity and purity of analyte peaks were confirmed using the ultraviolet (UV) spectra acquired for each peak by the PDA detector.

2. Experimental

2.1. Instrumentation

Chromatography was performed using Waters Associates chromatographic equipment (Milford, MA, USA) consisting of two 501 pumps, a Model 717 plus auto sampler, a Model 996 PDA detector, a pump control module, a SAT/IN module, and the program Millenium^{32} (version 3.05.01). An Alltech Ultrasphere C_{18} column (5 μm, 4.6×250 mm), fitted with a Waters guard column housing containing a Novapak C_{18} Guard Pak insert was used for all separations. A 0.5 μm high-pressure column prefilter [Alltech Associates (Aust.), Baulkham Hills, NSW, Australia] was used between the injector and the guard column. A Brinkmann SC/48R sample concentrator was used for drying deproteinised plasma extracts under nitrogen. A Model KS723 ISFET pH meter (Shindengen Electric MFG, Tokyo, Japan) was used for small volume sample pH adjustments.

2.2. Reagents

Creatinine, pseudouridine and cimetidine were all from Sigma (St. Louis, MO, USA). Sodium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate were both analytical reagents from AJAX chemicals (Sydney, NSW, Australia). Double deionized water was obtained from an IBC water purification system (Mansfield, QLD, Australia). HPLC-grade methanol was from Mallinckrodt (Paris, KY, USA).

2.3. Mobile phase

Eluent A was prepared by dissolving 0.78 g sodium dihydrogen orthophosphate and 0.71 g di-sodium hydrogen orthophosphate in 1 L of water (10 m*M* phosphate buffer), followed by filtering through a PTFE 0.45 μm filter (Millipore). The pH of eluent A was 6.8±0.1. Eluent B was methanol.

2.4. Standard solutions

Stock standard solutions of creatinine (10 mmol/L) and pseudouridine (10 mmol/L) were prepared in 0.1 *M* HCl. Stock internal standard solution (5 mmol/L cimetidine) was prepared by firstly dissolving the crystals in 10 mL of 0.1 *M* HCl followed by dilution up to 100 mL with water. A combination standard containing creatinine (0.35 mmol/L), pseudouridine (0.015 mmol/L) and cimetidine (0.25 mmol/L) was prepared by serially diluting appropriate volumes of stock solutions with the mobile

phase, and used for the quantitation of plasma samples. The internal standard solution added to each plasma sample was prepared by diluting cimetidine stock solution with mobile phase down to 0.25 mmol/L. All of the above solutions were frozen at −20 °C in small aliquots, and thawed daily as required.

2.5. Sample preparation

Blood samples were collected from 11 Belmont Red steers catheterised through the jugular vein. During blood collection, each catheter was flushed with 5 mL of heparinized saline (5 IU/mL 0.9% saline) using a polypropylene 10 mL syringe (Terumo Medical, Elkton, MD, USA). Five mL of blood were drawn through the catheter using the same syringe and then both blood and syringe were discarded. Then 8 mL of blood were drawn using a clean polypropylene syringe and the blood expelled slowly into an evacuated, heparinized test tube (Vacutainer, Beckman Scientific, Franklin Lakes, NJ, USA), where the blood was mixed gently and the tube placed on ice until centrifugation. Plasma was separated from the blood by refrigerated centrifugation (4 °C, 1200 *g*, Beckman J-6M, Gladesville, NSW, Australia) for 25 min. Plasma was then drawn from the samples using Pasteur pipettes and placed into 5 mL polypropylene sample vials and frozen at −20 °C.

Plasma samples were thawed and 600 μL pipetted into a 10 mL polypropylene centrifuge tube. A 100 μL aliquot of 0.25 mmol/L cimetidine (internal standard) and 2 mL of acetone were added to the centrifuge tube as well, the contents vortex mixed and allowed to stand at room temperature for 15 min before centrifugation at 5000 *g* for 15 min. The supernatant was decanted to a glass vial, and solvent evaporated under nitrogen at 35 °C. The residue was reconstituted with 240 μL mobile phase and its pH adjusted, with a small volume of HCl, to be between 6.5 and 7. The reconstitute was then transferred to an Eppendorf tube and frozen at −20 °C until HPLC analysis. The extracts were thawed when required, centrifuged at 5000 *g* for 5 min, and 50 μL of the supernatant were used for quantitation. Samples were prepared in duplicate.

2.6. Chromatographic conditions

Chromatography was performed at ambient temperature. Flow-rate used was 1 mL/min. The column was equilibrated for 10 min with eluent A prior to injection of each sample or standard. Both linear (curve 6) and step (curve 11) gradients were used in the gradient protocol:

Time	% A	% B	curve
0	100	0	*
2	100	0	11
12	0	100	6
15	100	0	11

The HPLC system used in this study had a lag time (the time taken for the solvents to travel from the solvent mixer/gradient maker to the column) of 6 min with a flow-rate of 1 mL/min. Thus, in the chromatogram, the change in composition from 100% eluent A to 100% eluent B is evident at about 8 min even though in the gradient table the change occurs at 2 min. Depending upon the lag time of each chromatographic system, then, the gradient shown here should be altered to obtain a similar performance. The PDA detector was used to collect chromatographic data from 210 to 350 nm.

2.7. Qualitative and quantitative analysis

Qualitative analysis was performed by spiking samples with the appropriate standard to observe the growth of the peak, and by comparing the ultraviolet (UV) spectra of sample peaks with those of standard peaks. Peak purity calculations were used to ensure that the analyte peak was free from co-eluting impurities with different spectral properties.

Quantitative analysis was performed by injecting a range of volumes (to cover the expected concentrations of analytes in the extracts) of the combination standard with each batch of samples chromatographed. A five-level linear calibration curve was constructed from the standard data and samples were quantitated using this calibration curve. Quantitation was performed by the internal standard method to compensate for the sample losses during extraction

and analysis. Quantitation of creatinine and pseudouridine were carried out at 262 nm, and that of cimetidine at 220 nm. Samples were prepared in duplicate in order to obtain true duplicate values for the concentrations of the analytes in them.

3. Results and discussion

Samples were de-proteinised using a ratio of sample volume to acetone that precipitated >99% of protein from plasma [11]. Although this was a simple extraction method, the drying time was lengthy (about 3 h) due to a large fraction of water that evaporated after the acetone. Forty-eight extracts were dried at a time in this study, so the lengthy drying time was not a handicap considering that the analyst's time was not required for these 3 h and per sample time was still very low compared to existing methods of extraction. In hindsight, however, the time to evaporate the de-proteinising agent could have been reduced by replacing acetone with acetonitrile because it is equally effective in deproteinisation [11] and, unlike acetone, acetonitrile forms an azeotrope with water and thus facilitates more rapid evaporation of water.

The extracts used for HPLC separations contained some particulate matter but the amount of this contaminant was minimised by careful pipetting of the extract. The extracts were not filtered as the volume was low and we wanted to keep the method simple. The pre-column filter and the guard column effectively protected the column, as evidenced by acceptable column performance after over 600 analyses, and they were replaced when the HPLC system pressure increased significantly (by about 100 p.s.i.). In the present study, this occurred after analysing about 70 extracts.

To further protect the column and pumps, the system was flushed with water for about 2 h and with methanol for 1 h after each batch of about 20 extracts and five standards. These precautions prevented pump problems and yielded consistent retention times for the large number of plasma samples analysed in this study.

Preliminary separations of random samples showed that the creatinine peak was much larger than the pseudouridine peak at the commonly used detection wavelengths of 254 and 220 nm; thus the sensitivity of pseudouridine was poor and would produce poor precision in the pseudouridine data. Because it was estimated from a few randomly chosen samples that the creatinine concentration in the plasma samples was approximately 25 times greater than that of pseudouridine, it was desirable to employ a detection wavelength at which the molar absorptivity of pseudouridine was approximately 25 times greater than that of creatinine. Under this constraint, it was found from the PDA spectra of both analytes that the optimum detection wavelength was 262 nm.

Cimetidine has been used as the internal standard for determination of creatinine only, and detected in a previous study [6] at 200 nm. However, its absorbance at our optimum wavelength of 262 nm was extremely low. The capability of the PDA detector enabled the chromatograms for cimetidine (internal standard) to be obtained at its λ_{max} (220 nm) while analytes of interest chromatographed on another wavelength (262 nm). Employing the λ_{max} as the detection wavelength for the internal standard reduced errors inherent in using alternative detection wavelengths located on a sloping region of the absorbance spectrum for cimetidine. This enabled us to maximise precision for the analyte data.

Excellent selectivities and sensitivities were achieved for creatinine, pseudouridine and cimetidine as shown for a typical plasma sample in Fig. 1. Peaks were identified by spiking the plasma extract with the standard for each analyte, and confirmed by comparison with standard spectra as shown in Fig. 2. Comparison of the spectrum at the peak apex to the spectra of the rest of the peak confirmed that the creatinine and pseudouridine peaks were free from co-eluting substances. Chromatograms of a typical plasma sample without the internal standard revealed that there were no possible interferents that could co-elute with cimetidine. Moreover, random purity checks of the cimetidine peak were performed to ensure the accuracy of the method. Since cimetidine is very strongly retained compared to the two analytes, a gradient elution scheme was required for the elution of all three compounds from the column. Without cimetidine, the run time could have been as short as 8 min, and an isocratic elution method (100% phosphate buffer)

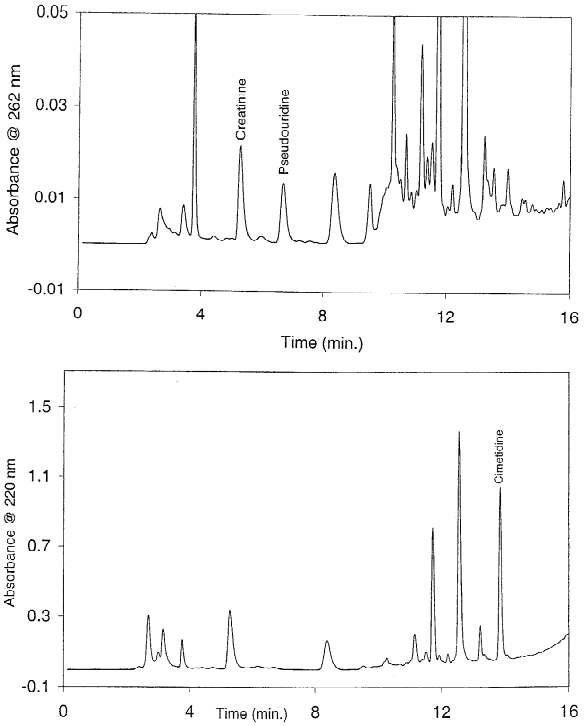

Fig. 1. Chromatograms of a typical plasma sample. The chromatogram processed at the 262 nm channel was used for quantitation of creatinine and pseudouridine, and that at the 220 nm channel for quantitation of the internal standard, cimetidine.

would have been sufficient to elute the two analytes of interest.

The common pH of HPLC analysis (samples, standards and the mobile phase) for creatinine has been pH 3 [6,9], pH 4 [12] or pH 4.9 [13]. Considering that the pKa_1 of creatinine is 3.55 [14] or 4.8 [15], a considerable amount of creatinine (as high as 50%) would have been undetected in these studies. As the pKa_2 of creatinine is 9.2 [15], our HPLC analyses (including samples, standards and the mobile phase) were performed within a pH range of 6.5 to 7, within which creatinine elutes as a single chemical form and is, therefore, detected as a single, narrow peak. As the pKa_1 and pKa_2 of pseudouridine are 9 and >13 [15], pseudouridine also elutes as a single chemical form within pH 6.5 to 7.

Calibration was linear up to 3.5 μmol for creatinine and 0.15 μmol for pseudouridine ($Y = 1.37e^4X - 3.22e^3$, $R^2 = 0.999$ for creatinine and $Y = 4.75e^5X - 2.95e^3$, $R^2 = 0.999$ for pseudouridine).

Table 1
Recovery of standards added to plasma samples

Compound	Addition[a] (nmol)	Mean recovery[b] (%) (±SD)
Creatinine	0	
	66.4	98.0 (±3.79)
	133	96.6 (±1.65)
Pseudouridine	0	
	2.90	96.3 (±4.97)
	5.80	101 (±1.26)

[a] Each level of addition was replicated five times ($n = 5$).
[b] Calculated as % recovery = 100[(amount observed − original amount)/amount added)].

Accuracy of the method was estimated by adding two levels of standard creatinine and pseudouridine to a sample and determining the amounts of standard recovered. Results are shown in Table 1. Although the precision of the method for both analytes is poor at lower concentrations, on average the recovery was over 96%. Detection limits were estimated at a signal-to-noise ratio of 3:1, which corresponds to 0.28 nmol for creatinine and 9.0 pmol for pseudouridine. They are comparable to the detection limits obtained by other researchers for these two analytes [9]. Mean (±SD) creatinine and pseudouridine values obtained were 59.5 (±2.79) and 2.28 (±0.103) μmol/L, respectively, for 248 samples from 11 animals. The values are consistent with the ranges reported in the literature [16,17], but with enhanced precision. Creatinine values were significantly lower compared with Jaffé method, which is also the trend observed when an electrophoretic method was compared with Jaffé method [18].

4. Conclusion

An accurate, robust, rapid and precise method was developed for the simultaneous determination of creatinine and pseudouridine in bovine plasma using cimetidine as an internal standard. Chromatographic conditions were developed so that comparable sensitivities and precisions were obtained for both analytes of interest despite the large differences in their concentrations in plasma. The importance of the pH of samples and mobile phase for both the accuracy and quantitation of these metabolites is

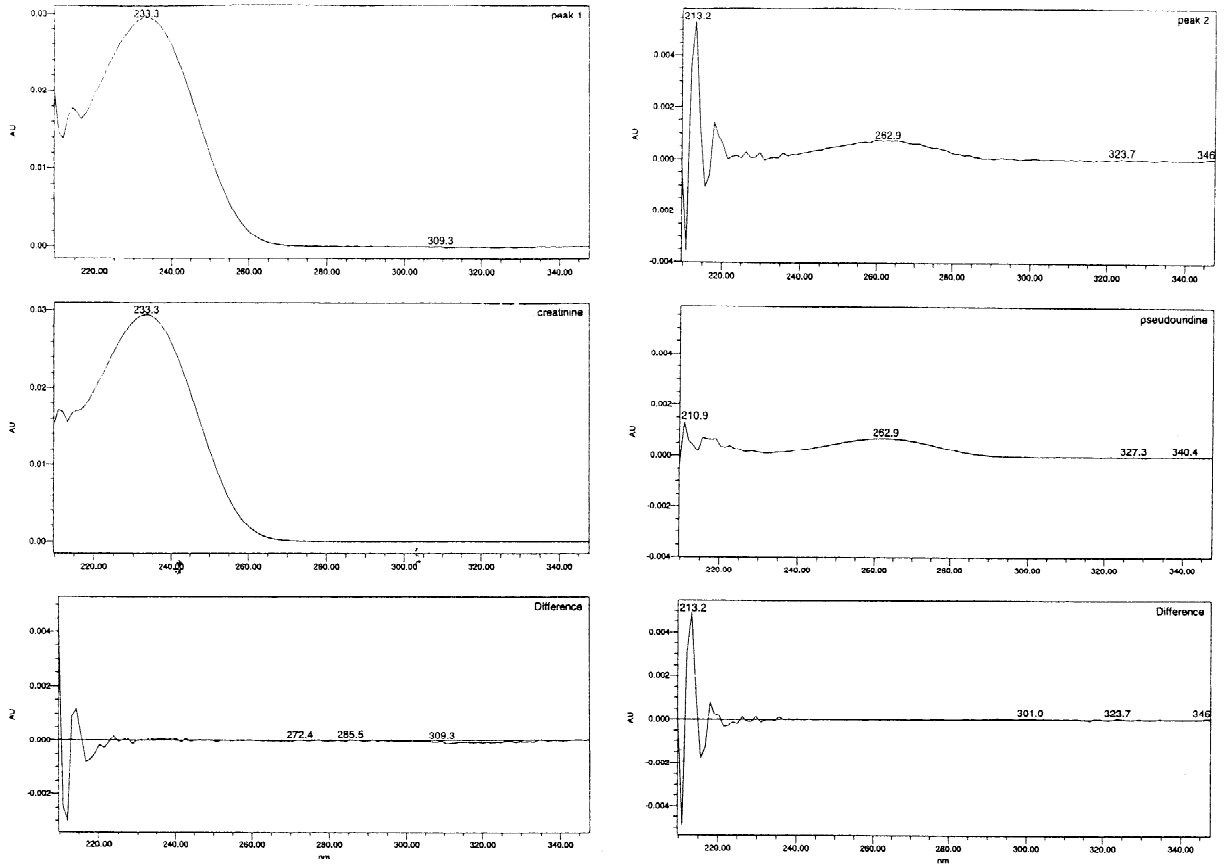

Fig. 2. Comparison of the UV spectra of creatinine (left) and pseudouridine (right) standards with corresponding peaks identified in a typical plasma sample. The three spectra shown in each case are in the order: spectrum of the identified peak, spectrum of the standard compound, difference spectrum between the identified peak and the standard compound.

emphasized, and recommendations are presented. This method will assist researchers interested in obtaining accurate values for pseudouridine and creatinine in bovine plasma as it is not affected by chromogenic interference as occurs with the Jaffé reaction [19].

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