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Simultaneous Liquid Chromatographic Determination of Creatinine and Pseudouridine in Bovine Urine and the Effect of Sample pH on the Analysis

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A rapid, reliable method for the simultaneous determination of creatinine and pseudouridine is described. Both analytes were detected at an optimum wavelength of detection (262 nm), considering the relative levels present in bovine urine. Cimetidine was used as the internal standard and detected at its maximum wavelength of absorption (220 nm) on a separate channel. All three compounds were eluted within 15 min, using a 10 mmol/L phosphate buffer (pH 6.8)-methanol gradient on a C18 column. Creatinine data were found to be significantly dependent upon the pH of the sample. Recoveries of both analytes were above 96%. Lowest detectable levels of creatinine and pseudouridine were 0.28 nmol and 9.0 pmol, respectively. The use of internal standard resulted in a method with high precision (standard deviation of 1.42 mmol/L and 0.027 mmol/L for creatinine and pseudouridine), yet one that was simple and rapid.

KEYWORDS: Creatinine; pseudouridine; HPLC; diode-array; internal standard; cimetidine

INTRODUCTION

Creatinine and pseudouridine are catabolites indicative of muscle mass (*1*) and protein synthesis (*2*), respectively. Creatinine is a byproduct of the creatine kinase reaction that dephosphorylates creatine phosphate as a mechanism of short-term energy repletion. It is formed from the spontaneous cyclization of creatine after the dephosphorylation reaction and is produced in a constant ratio to muscle mass (*3*). Pseudouridine (5-ribosyluracil) is a byproduct of the degradation of transfer and ribosomal ribonucleic acids and is also not salvaged by the body once released from the tissues in ruminants (*4*). As a result, it is used as an indicator of RNA turnover and hence protein synthesis (*5*).

Because creatinine and pseudouridine are established metabolic indicators, they are used in ruminant metabolism studies to indicate muscle mass and protein synthesis, respectively, in vivo. Urinary catabolites such as pseudouridine are often expressed in a ratio to creatinine because creatinine excretion is indicative of renal filtration rate, which decreases during dietary protein restriction as the ruminant seeks to conserve nitrogen (*6*). The ratio of pseudouridine to creatinine in 24 h urine collections has been shown to reflect changes in protein synthesis status of ruminants during growth, pregnancy, or lactation (*4*, *7*).

In recent years, the method of choice for the measurement of creatinine and pseudouridine has been reversed-phase high-performance liquid chromatography (RP-HPLC). Recently developed HPLC methods were available for the measurement of creatinine in human serum and urine (*8*), for the measurement of pseudouridine in human urine (*9*, *10*), and for the measurement of both creatinine and pseudouridine in bovine urine (*11*). However, these methods consisted of lengthy run times and complicated HPLC procedures, no internal standard, and mobile phases at pH values where more than one chemical form of creatinine would exist in samples. The methods available also lacked sensitivity for pseudouridine relative to creatinine in samples, because the detection wavelength was not optimized to achieve comparable sensitivities for both analytes. Furthermore, most methods were only applied to human urine rather than bovine urine. Recently, we developed a method to simultaneously determine creatinine and pseudouridine in bovine plasma (*12*), and in this paper we modified that method to determine these catabolites in bovine urine. The accuracy and precision of measuring these catabolites in urine has been increased through the use of optimum wavelengths for analytes and the internal standard with a photodiode array (PDA) detector and by the adjustment of sample pH to neutrality (6.5−7). However, sample preparation and analysis remain simple and reliable. Further confidence in the method was demonstrated by confirming the identity and the purity of analyte peaks with the UV spectra acquired for each peak by the PDA detector.

MATERIALS AND METHODS

1. Instrumentation. Chromatography was performed using Waters Associates chromatographic equipment (Milford, MA), consisting of two 501 pumps, a model 717 plus auto sampler, a model 996 photodiode array detector, a pump control module, a SAT/IN module, and the software program Millenium^{32} (version 3.05.01). A Waters Spherisorb ODS2 reversed-phase column (5 μm, 4.6 × 250 mm) was used for all

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Table 1. Gradient Table Used for Elution of Creatinine, Pseudouridine, and Cimetidine and Re-Equilibration of Column with Initial Chromatographic Conditions[a]

time	%A	%B	curve
0	100	0	
2	100	0	11
5	0	100	6
10	100	0	11
20	100	0	11

[a] Solvent A = 10 mM phosphate buffer at pH 6.8, solvent B = methanol, curve 6 = linear variation, curve 11 = step variation, flow rate = 1 mL/min, lag time = 6 min.

separations. A model KS723 ISFET pH meter (Shindengen Electric MFG. Co., Ltd, Tokyo, Japan) was used for small volume sample pH adjustments.

2. Reagents. Creatinine, pseudouridine, allantoin, sodium salt of uric acid, and cimetidine were obtained from Sigma Chemicals Co. (St. Louis, MO), while sodium dihydrogen orthophospate and di-sodium hydrogen orthophosphate were both analytical reagents from AJAX chemicals (Sydney, Australia). Double deionized water was obtained from an IBC water purification system (Mansfield, QLD, Australia), and HPLC grade methanol was obtained from Mallinckrodt (Paris, KY).

3. Mobile Phase. Eluent A was prepared by dissolving 0.78 g of sodium dihydrogen orthophosphate and 0.71 g of di-sodium hydrogen orthophosphate in 1 L of water (10 mmol/L), followed by filtering through a PTFE 0.45-μm filter (Millipore Corporation, Bedford, MA). The pH of eluent A was 6.8 $\pm$ 0.1. Eluent B was 100% methanol.

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 first 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 (0.7 mmol/L), pseudouridine (0.03 mmol/L), and cimetidine (0.5 mmol/L) was prepared by diluting appropriate volumes of stock solutions with the mobile phase and used for the quantitation of urine samples. The internal standard solution added to each urine sample was prepared by diluting cimetidine stock solution to 1.4 mmol/L with mobile phase. All of the above solutions were frozen at -20 °C as small aliquots and thawed daily as required.

5. Sample Preparation. Urine was collected from 11 Belmont-Red $\times$ Brahman crossbred steers (approximately 1 year old, mean weight 340 $\pm$ 13 kg standard deviation (SD)) housed in metabolic crates. Urine was collected into 20 L capacity plastic reservoirs of known weight, and 75 mL of 50% sulfuric acid was added into each reservoir prior to collection as a preservative. After 24 h of collection, reservoirs for each steer were weighed and their contents well mixed before taking a 100-mL sample for each animal. The pH of the sample was estimated using litmus paper (Whatman), and the sample was frozen at -20 °C until further analyses. The remainder of the urine was then discarded, and the reservoirs were washed and air-dried for reuse the following day. Urine was collected for a total of 6 days to obtain a representative sampling of catabolite excretion in each animal.

Immediately prior to catabolite analysis, urine samples were thawed and agitated, and 100 μL was transferred, using a pipet, into an Eppendorf tube. Then, 100 μL of 1.4 mmol/L cimetidine (internal standard) was added and the sample diluted to 1 mL with 800 μL of mobile phase. Each sample was then vortex mixed and filtered through a 13-mm, 0.45-μm, Polypure filter (Alltech Associates, Deerfield, IL), and the last 250 μL of filtrate was collected for HPLC analysis. The pH of the filtrate was checked and adjusted to be within pH 6.5$-$7 if found beyond this range. An injection volume of 30 μL was used for quantitation of creatinine and pseudouridine.

6. Chromatographic Conditions. Chromatography was performed at ambient temperature using a flow rate of 1 mL/min. The column was equilibrated for 10 min with eluent A prior to injection of each sample or standard. Following injection of each sample/standard, analytes were separated using the gradient shown in **Table 1**.

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 at a flow rate of 1 mL/min. Thus, in the chromatograms, the change in composition from 100% eluent A to 100% eluent B is evident at about 8 min, even though the gradient table change occurs at 2 min. Depending on the lag time of each chromatographic system, therefore, the gradient shown in **Table 1** should be altered to obtain a similar performance.

The chromatographic data were collected using the PDA detector at wavelengths between 210 and 350 nm, inclusive.

7. Qualitative and Quantitative Analysis. Qualitative analysis was performed by spiking the sample with appropriate standard to observe the growth of the peak and by comparing the UV spectra of sample peaks with those of standard peaks. Peak purity was evaluated to ensure that each analyte peak was free from coeluting impurities.

Quantitative analysis was performed by injecting a range of volumes of the combination standard (to cover the expected concentrations of analytes in the extracts), with each batch of samples analyzed on the HPLC. A 5-level calibration curve was constructed from the standard data, and samples were quantitated using this curve. Quantitation was performed using an internal standard, cimetidine, to compensate for the sample losses during extraction and analysis. Quantitation of creatinine and pseudouridine was performed at 262 nm, and that of cimetidine at 220 nm on duplicates of each sample.

RESULTS AND DISCUSSION

Creatinine, pseudouridine, allantoin, and uric acid standards were used to develop initial HPLC parameters for the method at a detection wavelength of 254 nm. Both allantoin and uric acid completed elution before creatinine and pseudouridine, so no interference was expected in the sample chromatograms. These preliminary separations of randomly selected samples also showed that the creatinine peak was much larger than the pseudouridine peak at the commonly used detection wavelengths of 254 and 220 nm. Because the sensitivity of pseudouridine at these wavelengths was poor, detection at these wavelengths was expected to produce poor precision in the pseudouridine data. Analysis of initial urine samples showed that their creatinine concentrations were approximately 25 times greater than their concentrations of pseudouridine, a factor similar to that observed in plasma (*12*). Therefore, the same optimum wavelength (262 nm) was used as in our previous study (*12*), at which the molar absorptivity of pseudouridine is approximately 25 times greater than that of creatinine. At the detection wavelength of 262 nm, comparable peak sizes and precisions were obtained for both creatinine and pseudouridine; hence, meaningful values for the ratio of pseudouridine to creatinine were obtained. Cimetidine was used as the internal standard, as it has been used by others (*8*) as an internal standard for creatinine at a detection wavelength of 200 nm. Its absorbance at our optimum wavelength of 262 nm was extremely low, however, but the use of a PDA detector enabled the cimetidine to be detected as the internal standard at its λ_{max} (220 nm), while the analytes were chromatographed at 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 and enabled us to maximize precision for the analyte data (*12*).

Review of the literature showed that the pH of HPLC analysis (samples and the mobile phase) for creatinine has been pH 3 (*8*, *11*), 4 (*13*), or 4.9 (*14*). The pK_{a1} values reported for creatinine are 3.55 (*15*) and 4.8 (*16*), and the pK_{a2} value reported is 9.2 (*16*). Therefore, the changes in chemical equilibria of creatinine with pH can be depicted as follows:

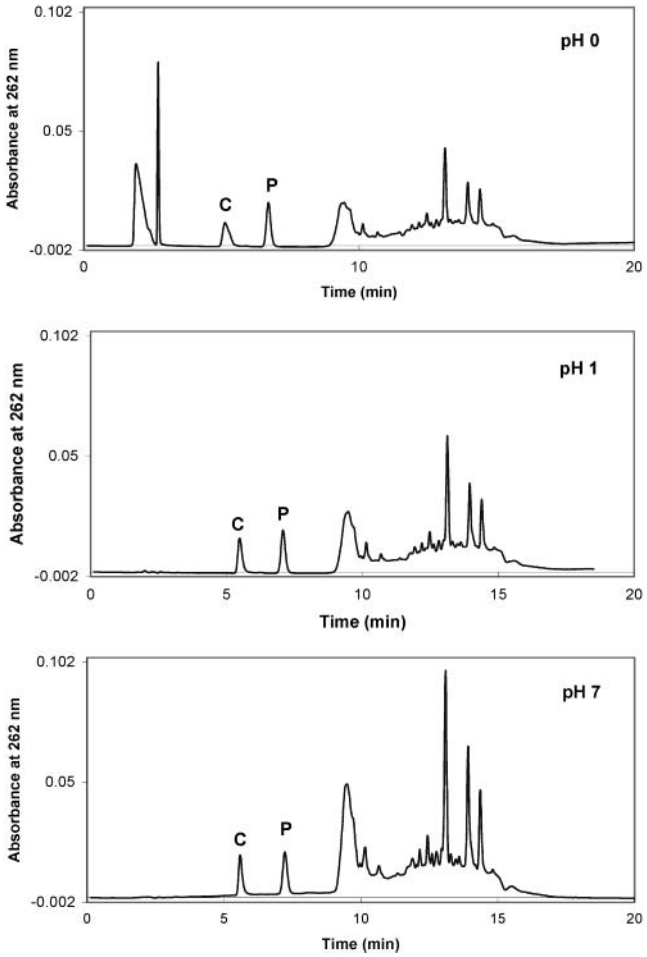

Figure 1. Effect of pH on the chromatograms of a standard solution of creatinine and pseudouridine at concentrations similar to those in the combination standard. All chromatographic conditions including mobile phase pH are as described in the materials and methods section. C = Creatinine, P = pseudouridine.

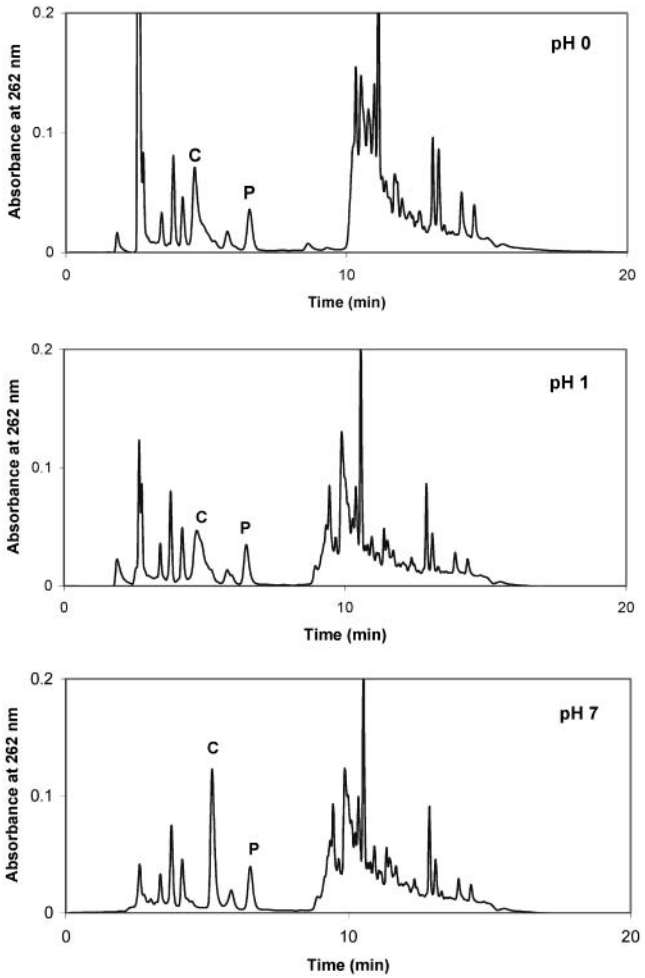

Figure 2. Effect of pH on the chromatograms of a typical urine sample. All chromatographic conditions including mobile phase pH are as described in the materials and methods section. C = Creatinine, P = pseudouridine.

$$C_4H_9N_3O^{2+} \leftrightarrows C_4H_8N_3O^{+} + H^{+}$$

within the pH range 2.8−6.8 (assuming $pK_{a1} = 4.8$)

$$C_4H_8N_3O^{+} \leftrightarrows C_4H_7N_3O + H^{+}$$

within the pH range 7.2−11.2 ($pK_{a2} = 9.2$)

Considering the pH of analysis used in earlier work shown above (*8*, *11*, *13*, *14*) and the differences in reported pK_{a1}, up to 50% of creatinine in the sample would have been doubly charged and the rest singly charged. As the mobile phase pH is also low (3−4.9), there is no chance of the doubly charged form being converted to the singly charged form during the passage through the column. As only one peak is used for quantitation, the error associated with creatinine data would have been significantly increased unless the two forms coeluted and have had the same molar absorptivity at the wavelength of detection.

The HPLC analyses (including samples, standards, and the mobile phase) in our method were performed within the pH range of 6.5−7, a range in which creatinine elutes as a single chemical form (singly charged) and is therefore detected as a single, narrow peak. As the pK_{a1} and pK_{a2} of pseudouridine are 9 and greater than 13 (*16*), respectively, pseudouridine also elutes as a single chemical form within the pH range 6.5−7.

To substantiate the effect of pH on the quantitation of creatinine, we adjusted the pH of the standard solution and that of a typical sample to 0, 1, and 7 by adding small amounts of HCl or NaOH and chromatographing with our normal mobile phase of pH 7. **Figure 1** shows the effect observed with the standard, and **Figure 2** shows that with the sample. The early peaks at pH 0 standard are unknown, and we suspect them to be impurities in HCl. As the volume of injection is between 15 μL and 50 μL, and the retention volume is over 5 mL, the sample is diluted with a relatively large volume of the mobile phase (of pH 7), and so the doubly charged creatinine is converted to the singly charged form during the passage through the column. However, as sample pH value decreases, the fraction of doubly charged creatinine increases and so will the time required for this conversion to be completed. Therefore, when the sample pH is low, creatinine will exist in two forms that coelute, resulting in a broad peak. As the pH of the sample approaches 7, where there is only one form of creatinine (singly charged form), the peak becomes narrower. Similar trends are observed in both the standard and sample chromatograms. The effect of pH appears to be more prominent in the sample than in the standard, and this may be due to additional buffering capacity of the samples making the conversion to one form more difficult than in the standard. We observed that the sample pH needs to be at least about 3 for it to be able to convert all creatinine to a single form with the mobile phase of pH 7.

This experiment clearly demonstrates the importance of pH on the analysis. Even when a mobile phase of pH 7 is used, a significant error can be made to creatinine data if the sample pH is not above 6.5 because there are two forms of creatinine under one peak in an unknown ratio. When the mobile phase

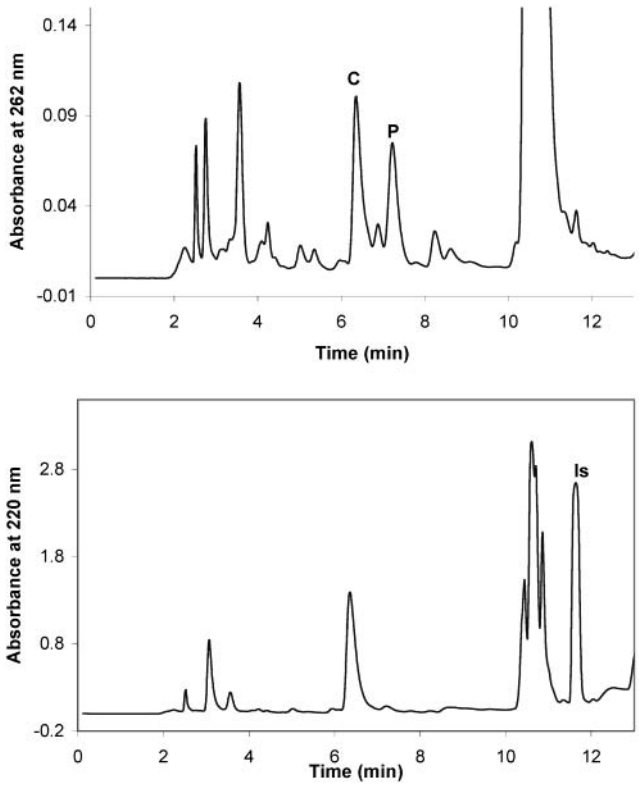

Figure 3. Chromatograms of a typical urine sample. Chromatogram processed at the 262 nm channel was used for quantitation of creatinine and pseudouridine, and that at the 220 nm channel was used for quantitation of the internal standard, cimetidine. C = Creatinine, P = pseudouridine, Is = Internal Standard.

pH is also low, as is the case with many studies (*8*, *11*, *13*, *14*), the error will be increased unless two forms of creatinine are individually quantitated and added together in moles. No further studies were performed on the effect of pH, as a thorough study of this nature is beyond the scope of an application paper such as this. However, the effect is clearly demonstrated in a qualitative manner so that the researchers are warned of the possible error unless the pH of samples, standards, and the mobile phase are adjusted to be within pH 6.5−7 prior to chromatographic analysis.

With the method presented in this paper, excellent selectivity and sensitivity were achieved for creatinine, pseudouridine, and cimetidine as illustrated in a chromatogram of a typical urine sample shown in **Figure 3**. Peaks were identified by spiking the urine with the standard for each analyte, and peak identities were confirmed by comparison with standard spectra (**Figure 4**). Spectral analysis of peaks confirmed that the creatinine and pseudouridine peaks were free from coeluting substances. RP-HPLC separation of a typical urine sample without the internal standard revealed that there were no interfering substances that could coelute with cimetidine, and this was confirmed by performing random purity checks of the cimetidine peak among samples analyzed to ensure the accuracy of the method.

Because cimetidine is more strongly retained than 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) would have been sufficient to elute the two analytes of interest.

Calibration was linear up to 140 nmols for creatinine and 6 nmols for pseudouridine ($Y = 1.54e^4X + 6.46e^3$, $R^2 = 0.999$ for creatinine and $Y = 5.73e^5X - 2.29e^4$, $R^2 = 0.999$ for pseudouridine). 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, and the results are shown on **Table 2**. Detection limits were estimated at a signal-to-noise ratio of 3:1, which corresponds to 0.28 nmols for creatinine and 9 pmols for pseudouridine. Typical mean (± SD) creatinine and pseudouridine values obtained were 11.8 (±1.42) mmol/L and 0.244 (± 0.027) mmol/

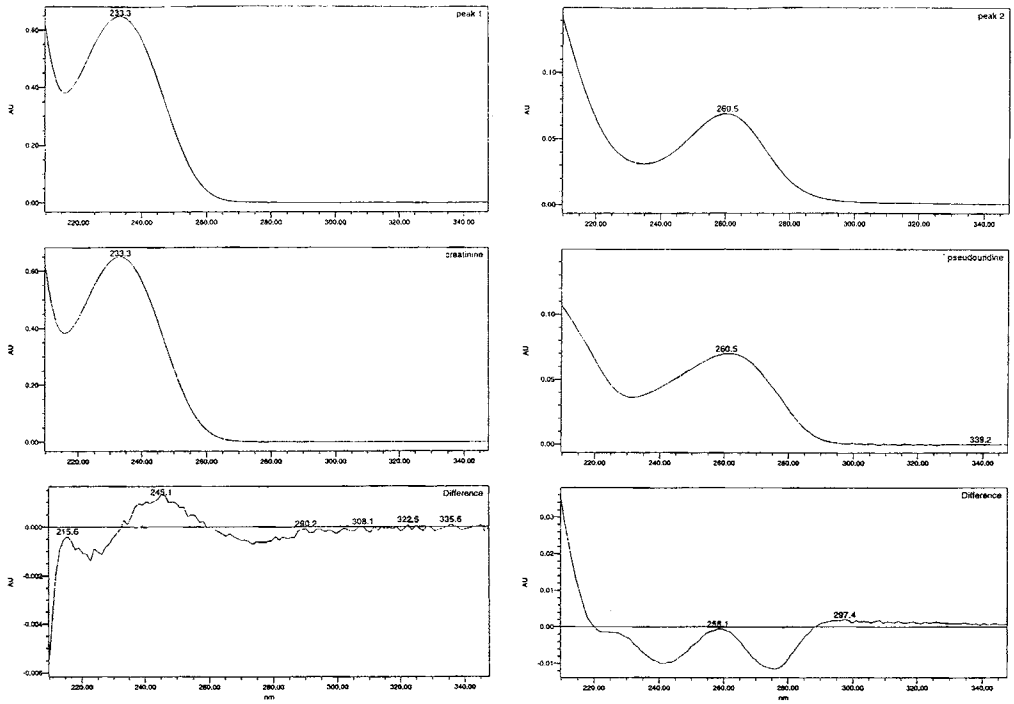

Figure 4. Comparison of the UV spectra of creatinine (left) and pseudouridine (right) standards, with corresponding peaks identified in a typical urine sample. Three spectra shown in each case are in the following order: spectrum of the identified peak, spectrum of the standard compound, and difference spectrum between the identified peak and the standard compound.

Table 2. Recovery of Samples Added to Diluted Urine Samples

compound	addition (μmol)[a]	%mean recovery[b] (±SD)
creatinine	0	
	35	96 (±2.2)
	70	96 (±1.2)
pseudouridine	0	
	1.5	99 (±0.53)
	3.0	99 (±0.42)

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

L, respectively, for 61 samples from 11 animals. The values were consistent with the ranges reported in the literature (*11*, *17*).

In recent years, HPLC has been the method of choice for the assay of creatinine and pseudouridine, as colorimetric methods such as the Jaffé method are prone to interferences from other substances in the matrix (*3*). However, as stated in the Introduction, most of the HPLC methods available to date were applications to human urine (*8*−*10*), in which the ranges of concentrations found differs from those in bovine urine. In comparing our method with the only method available for bovine urine (*11*), the limits of detection (LOD) were larger for creatinine (0.28 nmol in present study vs 0.06 nmol in ref 11) but comparable for pseudouridine (9 pmol vs 8 pmol). However, it should be noted that the LOD values in ref 11 were given in μmol/L; therefore, we assumed a volume of injection of 20 μL (volume of injection used for samples) to convert the LOD value to nmol and pmol. We prefer to express LOD values in mols rather than as concentrations, because extremely low LOD (concentration) values can be obtained by injecting large volumes. The large LOD for creatinine in our work reflects the relatively low sensitivity of creatinine signal at the wavelength of detection, which is well away from the λ_{max} of creatinine. As stated earlier, it was important to achieve comparable precision values for both analytes in this application. Wavelength of detection (262 nm) is selected considering relative concentrations of analytes in the samples so that both analytes give signals of similar sizes.

The selectivity of the method is superior to that of ref 11, as both analytes are well separated despite the reduced run times. This is particularly evident from the differences in precision for pseudouridine (0.027 in present study vs 0.215 mmol/L in ref 11). The partial overlap of the pseudouridine peak with an unknown peak of similar size in ref 11 must have contributed to the ambiguity in peak area, resulting in increased concentration variability, as reflected by the increased precision value. Precision for creatinine data was comparable in both studies (1.42 in present study vs 1.83 in ref 11). Run time for the elution of two analytes of interest in our method is considerably shorter (8 vs 40 min), and the chromatography is simpler (with no ion-pair reagents) than the previous method (*11*). The most important original feature in our method, however, is the enhanced accuracy and reliability of data achieved by using the appropriate pH for the analysis, by using an internal standard, and by peak purity checks using spectral data.

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