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Quantitative determination of urea concentrations in cell culture medium

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Abstract: Urea is the major nitrogenous end product of protein metabolism in mammals. Here, we describe a quantitative, sensitive method for urea determination using a modified Jung reagent. This assay is specific for urea and is unaffected by ammonia, a common interferent in tissue and cell cultures. We demonstrate that this convenient colorimetric microplate-based, room temperature assay can be applied to determine urea synthesis in cell culture.

Key words: urea determination, urea assay, urea synthesis, cellular urea, urea metabolism.

Résumé : L'urée est le principal sous-produit azoté du métabolisme des protéines chez les mammifères. Nous décrivons ici une méthode quantitative sensible de mesure de l'urée à l'aide d'un réactif de Jung modifié. Cet essai est spécifique à l'urée et n'est pas affecté par la présence d'ammoniac, une molécule qui interfère couramment dans les tissus et en culture cellulaire. Nous démontrons que cet essai colorimétrique commode, réalisé en micro-plaques à la température de la pièce, peut être utilisé pour mesurer la synthèse de l'urée dans les cultures cellulaires.

Mots-clés : mesure de l'urée, essai de détection de l'urée, synthèse de l'urée, urée cellulaire, métabolisme de l'urée.

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Introduction

Commonly used methods for urea determination are based on enzymatic and chemical assays. Enzymatic methods use the urea-metabolizing enzyme urease (Machado and Horizonte 1958), which degrades urea into ammonia. The produced ammonia is measured by a pH indicator (Orsonneau et al. 1992), ATP (Naslund et al. 1998), or H_2O_2 determination (Lespinas et al. 1989). However, a major disadvantage of these urease-based assays is that ammonia, which is often present in cellular and other biological samples, interferes with them (Morishita et al. 1997). Early chemical assays used diacetyl monoxime and required deproteination and heating to form colored product (Rosenthal 1955). By using *o*-phthalaldehyde and *N*-(1-naphthyl)ethylenediamine, Jung et al. (1975) improved this method and eliminated the need for heating and deproteination. The mechanism of this color reaction is not established but is likely to involve two-step reactions according to Jung et al. (1975). The first step is a specific condensation reaction of *o*-phthalaldehyde with urea. In the second step, the formed carbonium ion reacts with the coloring reagent *N*-(1-naphthyl)ethylenediamine to produce a colored product. However, while this method works for samples such as urine and blood that contain high urea concentrations, it is not suitable for samples containing low urea concentrations. Furthermore, the peak wavelength of the reaction product at 505 nm coincides with the phenol red absorbance maximum in acidic medium. These characteristics greatly limit this method's usefulness for applications involving low urea concentration samples, especially those that contain phenol red such as cell culture medium samples.

While developing an improved urea assay, we found that coupling the reaction to primaquine in place of *N*-(1-naphthyl)ethylenediamine greatly enhances the assay performance. In this paper, we demonstrate the effectiveness of this modified Jung reagent for urea determination of cell culture medium samples.

Materials and methods

Materials and instrumentation

All chemicals were of analytical purity and were obtained from Sigma Aldrich (St. Louis, Missouri). Clear flat-bottom Costar 96-well plates were purchased from VWR International. Optical density values and absorbance spectra were

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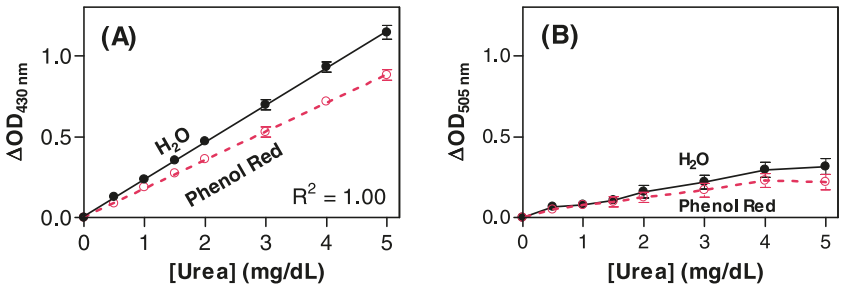
Fig. 1. Standard curves of urea diluted in water and in a phenol red medium (RPMI 1640 medium). Standard curves were generated using (A) *o*-phthalaldehyde/primaquine reagent and (B) the original Jung reagent (*o*-phthalaldehyde/naphthylethylenediamine).



recorded on a Molecular Devices SpectraMax 384 Plus microplate spectrophotometer.

Cell culture

Plasmodium falciparum (3D7 strain) was maintained in tissue culture flasks in phenol red containing RPMI 1640 culture medium supplemented with sodium bicarbonate (2 mg/mL), hypoxanthine (100 μmol/L), Albumax II (0.25%), and gentamycin (50 μg/mL) in a humidified incubator at 5% CO_2, 6% O_2, and 37 °C. Cultures were double-synchronized one cycle prior to the experiment. Synchronized cultures (2% parasitemia) were washed and resuspended in fresh, prewarmed medium at 1% hematocrit during the trophozoite stage (approximately 24 h postinvasion). A single parent culture was split into $N = 3$ flasks to serve as biological replicates. Culture medium samples were collected at 0 and at 8 h intervals thereafter. Media samples were purified of cells by passage through a 0.2 μm filter and frozen at –80 °C until analysis.

Urea assay

Urea reagents were prepared as described in Jung et al. (1975). The final Jung working reagent consisted of 100 mg/L *o*-phthalaldehyde, 215 mg/L *N*-(1-naphthyl)ethylenediamine, 2.5 mol/L sulfuric acid, 2.5 g/L boric acid, and 0.03% Brij-35. The modified reagent used 513 mg/L primaquine bisphosphate in place of the 215 mg/L *N*-(1-naphthyl)ethylenediamine reagent. The urea standard was prepared in double-distilled water and contained 5.00 mg/dL urea. To perform the assay, 50 μL of water, 50 μL of the 5.00 mg/dL standard, and 50 μL samples were transferred into separate wells of a clear flat-bottom 96-well plate. Then to each well, 200 μL of freshly prepared working reagent was added and mixed quickly by gently rocking the plate. The reaction was incubated for 1 h at room temperature. Optical densities (OD) at 430 and 505 nm were measured on the plate reader for assays using the modified reagent and the original Jung reagent, respectively.

The calibration curve (Fig. 1A) shows that the assay is linear between 0.00 and 5.00 mg/dL urea. For calculation of the sample urea concentration, the experimenter can choose either to use the slope of the standard curve or to use a single urea concentration (see below). We found that it is sufficient to use one blank (water) and one single urea concentration (5.00 mg/dL) to calculate the sample urea concentrations. In this work, urea concentration in the sample was calculated from the OD values:

$$[\text{Urea}] = \frac{\text{OD}_{\text{SAMPLE}} - \text{OD}_{\text{BLANK}}}{\text{OD}_{\text{STANDARD}} - \text{OD}_{\text{BLANK}}} \times n \times [\text{Standard}]\ (\text{mg/dL})$$

where $\text{OD}_{\text{SAMPLE}}$, $\text{OD}_{\text{STANDARD}}$, and OD_{BLANK} are $\text{OD}_{430\,\text{nm}}$ values of the sample, standard, and water blank, respectively. [Standard] is the concentration of the urea standard (5.00 mg/dL or 0.83 mmol/L) and n is the dilution factor. Dilution of samples in distilled water is necessary when sample $\text{OD}_{430\,\text{nm}}$ values are higher than the $\text{OD}_{430\,\text{nm}}$ value for the 5.00 mg/dL urea standard.

Results and discussion

To develop an improved Jung urea reagent, we compared the original Jung reagent *N*-(1-naphthyl)ethylenediamine with four commercially available chemical reagents that could potentially react with the isoindoline derivative in the Jung reaction: 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (primaquine), 1,3-dihydroxynaphthalene, 1,3,5-trihydroxybenzene, and 4,6-dihydroxy-2-aminopyrimidine. The reaction of the primaquine with urea and *o*-phthalaldehyde produced the strongest color change. The colored product had a peak at 430 nm on the absorbance spectrum. The reaction reached a plateau after 50 min at room temperature. With the original Jung reagent *o*-phthalaldehyde/*N*-(1-naphthyl)ethylenediamine, the reaction was slower and the colored product had a peak at around 505 nm. To compare these two reagent systems, we ran assays using urea standards diluted in water and in a phenol red medium (RPMI 1640 medium). Using primaquine (Fig. 1A), the standard curve was linear up to 5.00 mg/dL (0.83 mmol/L) urea, whereas color formation was much weaker with the original Jung method and the standard curve was not linear within this urea concentration (Fig. 1B). Based on the 3× standard deviation of six blanks, we determined a detection limit of 0.08 mg/dL (0.013 mmol/L) urea for the modified reagent and 0.46 mg/dL (0.077 mmol/L) urea for the original Jung reagent. The detection limit for the modified Jung method is much lower than urea concentrations in most biological samples. Furthermore, the results reveal that this modified assay can be performed in phenol red containing culture media, if phenol red (e.g., medium without serum) is included in the solutions used to construct the standard curve.

To validate this assay in cell culture medium, we assessed the effects of ammonia, an interferent in urease-based assays. Ammonia or its conjugate acid ammonium is usually present at 1–5 mmol/L (5.3–26.5 mg/dL) concentrations in

Fig. 2. Effect of ammonia on the urea assay. (A) Urea standards at concentrations of 0.00–10.0 mg/dL (1.67 mmol/L) were mixed 1:1 with distilled water (control) or with 10.0 mmol/L ammonium chloride. (B) A 2.00 mg/dL (0.33 mmol/L) urea standard solution was spiked with increasing concentrations of ammonium chloride as indicated on the figure. All assays were run in triplicate.

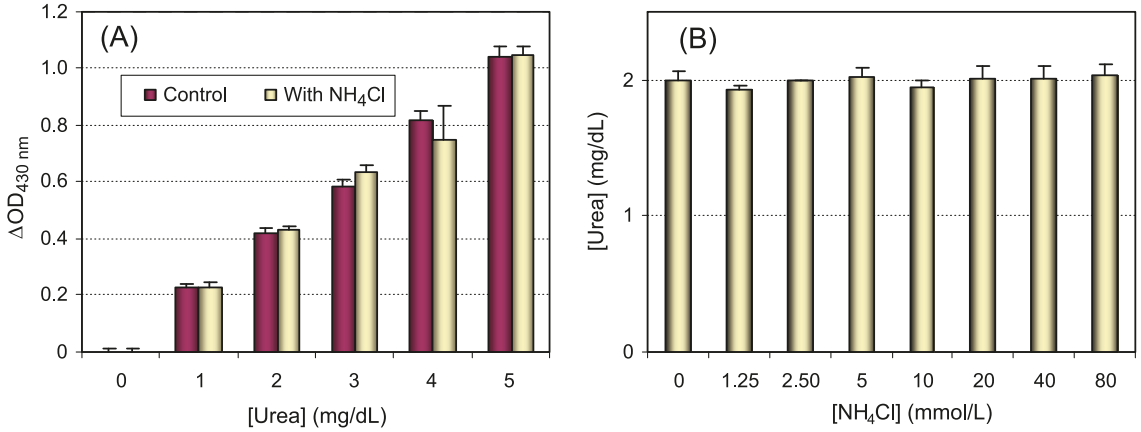
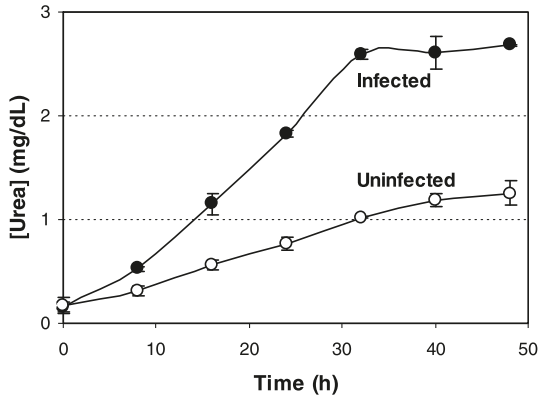


Table 1. Analytical recovery in a 24 h *Plasmodium falciparum* infected red blood cell culture with known amounts of spiked urea.

Spike (mg/dL)	Found (mg/dL)	Computed spike (mg/dL)	Recovery (%)
0.00	1.9±0.0	na	na
1.25	3.2±0.0	1.3±0.0	100±1
2.50	4.3±0.0	2.4±0.0	94±1
5.00	6.8±0.1	4.8±0.1	97±1

Note: A stock solution of urea was added to the culture medium sample. Spike shows the calculated final urea concentration added to the sample. Found lists the urea concentration in the samples determined with the modified Jung method. It represents the total urea in the original culture sample plus the added urea spike. Computed spike is the difference between the found and unspiked culture sample. Recovery is the percentage of computed spike over spike. Samples that were spiked with 5.00 mg/dL (0.83 mmol/L) urea were diluted 1:1 in distilled water (dilution factor $n = 2$) prior to assaying for urea concentration. na, not applicable.

Fig. 3. Time course of urea production in *Plasmodium falciparum* infected and uninfected red blood cell cultures. Data are presented as mean ± SD ($n = 3$).



most cell cultures (Nagao et al. 1989; Takagi et al. 2000; Aoyagi 2003). To assess the effect of ammonium on the current chemical assay, we performed the assay in the presence of 5.0 mmol/L (26.5 mg/dL) ammonium chloride with urea concentrations ranging from 0.00 to 5.00 mg/dL or from 0.00 to 0.83 mmol/L (Fig. 2A). No significant difference between the color reaction in the absence and presence of ammonium chloride was observed. In addition, we performed a titration of ammonium chloride added into a 2.00 mg/dL (0.33 mmol/L) urea standard and ran the urea assay. The results (Fig. 2B) show that this urea assay is not affected by 80.0 mmol/L ammonium or 242-fold in molar excess over urea, which is far higher than typical ammonium concentrations in cell cultures.

To further evaluate the suitability of this assay in culture medium, we determined the analytical recovery by spiking known amounts of urea into medium samples from *P. falciparum* infected red blood cell cultures. Assays were run to determine the total urea concentrations in the unspiked and spiked samples. As shown in Table 1, the spiked urea was quantitatively found in the cultured medium sample at recovery rates between 94% and 100% (Table 1), suggesting that the assay is compatible with this sample matrix.

Urea synthesis in both *P. falciparum* infected and uninfected red blood cell cultures was also determined using our urea assay method. As shown in Fig. 3, both infected and uninfected red blood cells secrete urea into the culture media, but with distinct kinetics. In the uninfected samples, a steady but slow increase in urea was observed that reached a plateau at 1.2 mg/dL (0.20 mmol/L) after 48 h. In contrast, when cells were infected with *P. falciparum*, urea production was accelerated. The rate was approximately double that in the control uninfected cells. Urea production reached a plateau earlier at around 32 h postinfection. Approximately twofold higher urea (2.7 mg/dL, 0.45 mmol/L) was produced in the infected cells than in the uninfected cells, as expected.

In conclusion, modifying the Jung urea assay by using *o*-phthalaldehyde/primaquine instead of *o*-phthalaldehyde/*N*-(1-naphthyl)ethylenediamine in the assay reagent allows quantitative, sensitive, and robust determination of urea in cell culture media. This assay is especially useful when ammonium chloride is to be used in cell cultures to study ammonia metabolism and urea biosynthesis (Nagao et al. 1989; Takagi et al. 2000; Aoyagi 2003).

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