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7 **Highly selective and sensitive determination of several antioxidants in human**
8 **breast milk using high-performance liquid chromatography based on Ag(III)**
9 **complex chemiluminescence detection**

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Abstract: Ascorbic acid (AA), uric acid (UA) and glutathione (GSH) are the most important water-soluble antioxidants. The concentrations of GSH and glutathione disulfide (GSSG) and their molar ratio are the indicators of oxidative stress. Little is known about the contents of UA, GSH and GSSG in human milk; a reliable and sensitive method to monitor the concentrations of the four compounds simultaneously in human milk is of critical importance. A new method for separation and quantification of these water-soluble antioxidants by HPLC coupled with Ag(III) chemiluminescence detector has been developed in this work with better recoveries. The antioxidants contents were determined in different times of lactation utilizing this method. The results show that the levels of AA, UA, GSH and GSH/GSSG of human colostrum are significantly higher than those of mature milk ($P < 0.05$). It is concluded that colostrum contains more water-soluble antioxidants than mature milk.

Keywords: Antioxidants; Chemiluminescence; Ag(III) complex; HPLC; Human breast milk

1. Introduction

Human breast milk (HBM) is an ideal and natural food for newborn babies. In addition to being the best source of nutrients, it supplies a number of defence components (Ali, Ozcan, Abdurrahim, Sahbettin, & Mehmet, 2006; VanderJagt, Okolo, Costanza, Blackwell, & Glew, 2001), containing many non-enzymatic antioxidants. The antioxidants include glutathione (GSH), ascorbic acid (AA), uric acid (UA), which are three of the most important water-soluble small molecular

antioxidants. Antioxidants in general can protect against oxidative damages, which are origins of many disease pathologies (Korir, Wachira, Wanyoko, Ngure, & Khalid, 2014; Granot, Golan, Rivkin & Kohen, 1999). The small molecular antioxidants in HBM help scavenge reactive species and reduce the oxidative damages of biological molecules.

Ascorbic acid (AA) in HBM, possessing a low reduction potential, usually reacts with radicals and oxidants rapidly. Thus, it is a critical component for the body's defence (Romeu Nadal, Morera Pons, Castellote, & L'opez Sabater, 2006). Uric acid (UA) is another effective scavenger in HBM for aqueous peroxide radicals (Ali, Ozcan, Abdurrahim, Sahbettein & Mehmet, 2006) and it can protect the integrity of an infant intestinal canal mucosa (Buescher & Mcilheran, 1988). Glutathione (GSH), a major intracellular non-protein thiol compound. The high nucleophilicity of the thiol group confers GSH a free radical scavenger both under physiological conditions and in xenobiotic toxicity (Matos, Ribeiro & Guerra, 2015). Under the pathological conditions, GSH is in equilibrium with its oxidized form GSSG. The concentrations of GSH and GSSG and their molar ratios can correlate with the levels oxidative stress in cells (Monostori, Wittmann, Karg & Túri, 2009). GSH is formed in cells, but the capacity of cells in the infants to synthesize GSH has not well developed, so GSH in HBM taken by infants will be the dominant source (Ankrah, Appiah Opong & Dzokoto, 2000). Undoubtedly, the simultaneous determinations of these antioxidants in HBM are important for the evaluation of the antioxidant capacity of HBM (Li, Hosseinian, Tsopmo, Friel & Beta, 2009).

Currently, there are few reports for the simultaneous determinations of AA, UA, GSH and GSSG. HPLC coupled with UV (Ferin, Pavão & Baptista, 2013; Fontannaz, Kilinc & Heudi, 2006; Kand'ár, Drábková & Hampl, 2011; Nadal, Pons, & Sabater, 2006), with fluorescent (Ferin, Leonor Povao, & Baptista, 2012), with mass (Squellerio, 2012), with electrochemical (Rose, & Bode, 1995; Li, & Franke, 2009; Vovk, Bogataj, Rožkar, Kmetec, & Mrhar, 2005), and with chemiluminescence (Bai, Chen, Lu, & Lin, 2013; McDermott, Terry, Conlan, Barnett, & Francis, 2011) detection methods have been developed for the determinations of these antioxidants.

Since GSH and GSSG contain no strong chromophores, UV detector is incompetent. For fluorescent detections, chemical derivatizations are usually required, and moreover, the pre- or post- column derivatizations often involve complicated and time-consuming protocols. HPLC-MS provides high selectivity and sensitivities, but requires special and expensive equipments. Electrochemical detector (ECD) is usually used in the determinations of small molecular antioxidants; major limitations of this approach include the poor reproducibility and the narrow applicability (Squellerio, 2012). Compared with the detection methods mentioned above, the CL technique provides higher sensitivities, lower detection limits, wider linear ranges, and lower costs. In recent years, the HPLC-CL method has been used in the determination of trace levels of compounds in complex matrices by use of the characteristic of CL detection.

In the early study, we found some antioxidants may lead to CL signal enhancement remarkably for the Ag(III) - luminol system. Ag(III) refer to

bis(hydrogen periodato)argentate(III) complex anion, its molecular formula is $[\text{Ag}(\text{HIO}_6)_2]^{5-}$. On the basis of the chromatographic and CL conditions optimized before, we have developed a method which is accurate, sensitive and is used to determine simultaneously AA, UA, GSH and GSSG in HBM for the first time.

2. Experiment

2.1. Instrumentation

The HPLC- CL detection system consisted of the HPLC system and a post-column CL detection system (shown in Fig. 1). The HPLC system is a Shimadzu LC-15C series (Shimadzu, Japan) equipped with two pumps, a thermostatic column compartment, a manual sample valve injector with a 20 μL loop, and an analytical column. The CL detection system is composed of a peristaltic pump, a model IFFS-A CL detector equipped with a glass coil (used as the reaction coil and the detection cell) and a photomultiplier tube (PMT, Beijing, China) that collects the CL emission.

2.2. Reagents

GSH and UA were obtained from Sigma-Aldrich. AA and GSSG were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Luminol and HPLC grade methanol were obtained from Merck (Darmstadt, Germany). Acetic acid, ethylenediaminetetraacetic acid disodium salt (EDTA), trichloroacetic acid (TCA), perchloric acid (PCA), metaphosphoric acid (MPA, 40%, w/v), sodium tungstate, metaphosphoric, AgNO_3 and KOH were

obtained from Beijing Chemical Reagent (Beijing, China) or Tianjin Chemical Reagent (Tianjin, China). All other chemicals used in this work were of analytical grade. A stock solution of 2.0×10^{-2} mol L⁻¹ luminol was prepared in 1.0 mol L⁻¹ NaOH and was kept in the dark at 4 °C. A [Ag(HIO₆)₂]⁵⁻ stock solution (0.030 mol L⁻¹) was prepared, and its concentration was determined spectrophotometrically at 362 nm using the molar absorptivity $\epsilon = 1.26 \times 10^4$ L mol⁻¹cm⁻¹. The stock solutions were diluted with water to obtain the appropriate concentrations of the working solutions.

2.3. Stock solutions

Stock solutions of about 0.01 mol L⁻¹ GSH, GSSG and AA were prepared daily in 0.5% PCA solution (v/v). The UA stock solutions were prepared in 0.01 mol L⁻¹ NaOH solution to give a final concentration of 0.01 mol L⁻¹. All stock stocks were stored in a refrigerator at -20°C and protected from light. The standard solutions were prepared by diluting the stock solutions with 0.5% (v/v) PCA solution.

2.4. Sample collections and preparations

Samples of HBM were collected using a manual breast pump from 126 postpartum women aged between 25 and 35 years old with normal, spontaneous full term delivery. HBM were collected into opaque bottles and immediately placed on ice. Before pre-treatments, the milk samples were shaken with vortex turbulence. Approximately 0.5 mL of HBM samples were placed in 1.5 mL Eppendorf tubes, and 0.5 mL of PCA

15% (v/v) containing 1 mmol L⁻¹ EDTA was added to each tube. The mixtures were shaken for 40 s and incubated on ice for 10 min to completely precipitate the milk proteins. The acidic suspensions were then centrifuged at 4 °C (10 min, 12000×g) and the supernatants were subjected to immediate analysis. The supernatants were diluted 5 to 20 times with 0.5% (v/v) PCA. All sample solutions were filtered through a 0.22 μm nylon membrane.

2.5 HPLC–CL detection conditions

AA, UA, GSH and GSSG were separated using a PLATISILTM ODS-C18 column (250 mm×4.6 mm, 5 μm) at 35 °C with a gradient elution program at a flow rate of 0.8 mL/min. The mobile phases consisted of (A) methanol and (B) acetic acid water solution (0.1%, v/v, pH 3). The gradient elution program was set at: 95% B (0–8 min), 95–90% B (8–10 min), 90% B (10–20 min). The post-column CL reagents, luminol and [Ag(HIO₆)₂]⁵⁻, were delivered together using a peristaltic pump at the rate of 2.2 mL min⁻¹ and merged at a T-piece 1. The stream was then mixed with the column eluate in another T-piece 2 located 2 cm from the entrance of the detector. The potential of the photomultiplier tube was set as -600 V.

3. Results

3.1 Optimization of sample preparation

AA, UA, GSH and GSSG are stable in acidic medium. We investigated some acidic deproteinization reagents including TCA, PCA, MPA and TFA. The experimental

results showed that MPA was not appropriate for GSH and GSSG extraction, and TCA was not appropriate for AA extraction. PCA was the most suitable precipitating reagent because it gave the strongest CL signal for all the analytes. Geoffrey and coworkers have reported that when PCA was used as the deproteinization reagent, it did not result in significant GSH oxidation during sample preparation (McDermott, Terry, Conlan, Barnett, & Francis, 2011). PCA concentrations ranging from 1% to 15% (all contained 1 mmol L⁻¹ EDTA) were investigated, showing that 10% PCA was the most suitable concentration for all the four analytes. Thus, the 10% PCA was the optimized deproteinization reagent used in the present study.

3.2. Optimization of chromatographic conditions

For the HPLC-CL method, the selection of the mobile phase was a key factor. The mobile phase should not only be suitable for optimal separation of analytes but also compatible with the CL reaction. Several compositions of the HPLC mobile phases were tested such as methanol-sodium dihydrogenphosphate, methanol-acetic acid and acetonitrile-phosphoric acid. We found that acetonitrile caused heavy quenching of the CL signal in the luminol-[Ag(HIO₆)₂]⁵⁻ system. Although the mobile phases of both methanol-sodium dihydrogenphosphate and methanol-acetic acid buffers could enhance the CL signal of luminol-[Ag(HIO₆)₂]⁵⁻-antioxidant systems, the methanol-acetic acid mobile phase gave the highest signal-to-noise (S/N) ratios.

The flow rates of the mobile phase were also investigated from 0.6 - 1.0 mL min⁻¹, and 0.8 mL min⁻¹ was chosen as the best flow rate when the S/N ratios were

considered. Due to the increasing methanol content in the mobile phase solution, the CL baseline signals were stable while the CL signals of the analytes increased significantly. Therefore, a gradient elution program was selected for the separation of the analytes. Under the optimized conditions, the four analytes were baseline separated and detected in less than 14.0 min.

3.3. Optimization of CL detection

To improve the sensitivity of the CL detection, the CL conditions were examined and several parameters were optimized. The concentrations of luminol were tested over the range 0.1 - 1.0 $\mu\text{mol L}^{-1}$. The CL intensity and noise increased with increasing luminol concentrations. And the optimum S/N ratio was observed at a luminol concentration of 0.2 $\mu\text{mol L}^{-1}$. The effect of the $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ concentration on the intensities was investigated in a range of $5.0 \times 10^{-5} \mu\text{mol L}^{-1}$ to $2.0 \times 10^{-4} \mu\text{mol L}^{-1}$, demonstrating that when the $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ concentration was $(1.0-1.2) \times 10^{-4} \mu\text{mol L}^{-1}$, almost all the analytes gave the strongest CL signals. The NaOH concentrations in the $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ solution was studied over a range of 0.05 to 3.0 $\mu\text{mol L}^{-1}$; the CL intensities of all the analytes gradually increased with increasing [NaOH] up to 0.15 mol L^{-1} , where a maximum CL signals were reached. Further increase of [NaOH] resulted in decreases in the CL signals. Thus, 0.15 mol L^{-1} [NaOH] in $1.0 \times 10^{-4} \text{mol L}^{-1}$ $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ solutions was chosen for this work.

The effects of the CL reagents flow rate on the relative CL intensities of the four analytes were studied over the range of 1.5 to 4.5 mL min^{-1} . The results showed that

the relative CL intensities increased with increasing in flow rate from 1.5 to 2.2 mL min⁻¹, and beyond 2.2 mL min⁻¹, the relative CL intensities did not increase significantly. The optimized results are summarized in Table S1 in the Supplementary Material.

3.4 The analytical performance of the developed HPLC-CL system

The performance of the method was evaluated under optimal detection conditions. The linear ranges were 0.5-20 µmol L⁻¹ for AA, 0.1-20 µmol L⁻¹ for UA, 0.25-10 µmol L⁻¹ for GSH and 0.1-10 µmol L⁻¹ for GSSG, respectively. The detection limits (S/N=3) for the above compounds were 4.60×10⁻⁸ mol L⁻¹, 6.44×10⁻⁸ mol L⁻¹, 4.85×10⁻⁸ mol L⁻¹ and 3.88×10⁻⁸ mol L⁻¹, respectively. The precision of the method was evaluated by analyzing 7 replicates of the standard solution containing 5×10⁻⁶ mol L⁻¹ of each target compound. The linear ranges, linear equations, detection limits, relative standard deviations (RSD), and other variables derived from the experiments are summarized in Table 1.

3.5 Application to human milk samples

The optimized method was applied to the analysis of AA, UA, GSH and GSSG in HBM. The identifications of compounds were performed by CL detector based on the relative retention times of the compounds. Figure 2 shows the typical chromatograms of the samples of HBM and the samples were spiked with the four compounds. Good relative recoveries (from 91.80% to 106.7%) were obtained, see Table 2.

Following the procedure described in Section 2.4, the proposed method was successfully applied to the analysis of human colostrum and mature milk from 106 postpartum women aged between 25 and 35 years old. Colostrum samples were collected on the 3rd to 7rd day after delivery and mature milk samples were collected between 7th days to 12th months after delivery. The antioxidant capacities of colostrums of lactating mothers in China are 163.1-453.3 $\mu\text{mol L}^{-1}$ for AA, 58.15 - 96.15 $\mu\text{mol L}^{-1}$ for UA, 2.79-77.2 $\mu\text{mol L}^{-1}$ for GSH and 0.75-6.49 $\mu\text{mol L}^{-1}$ for GSSG. In contrast, these are 77.6-510 $\mu\text{mol L}^{-1}$ for AA, 30.6-86.8 $\mu\text{mol L}^{-1}$ for UA, 2.38-45.38 $\mu\text{mol L}^{-1}$ for GSH and GSSG 0.34-4.43 $\mu\text{mol L}^{-1}$ for GSSG from the mature milk. The results are also given in Table 3.

4. Discussion

AA, UA and GSH are the most important water-soluble antioxidants. Using our proposed method, we collected 40 colostrums and 48 mature milk samples. The subjects are healthy who have no acute or chronic illness including gestational diabetes, hypertension of pregnancy and pharmacotherapy including vitamin supplementations. The data of the antioxidant capacities were analysed statistically by rank test, using SPSS 19.0. The statistic results show that the contents of AA, UA, GSH and the ratio of GSH and GSSG in mature milk are significant ($P < 0.05$) lower than those in colostrum. Moreover, the concentrations of AA ($P = 0.001$, $r = 0.608$), UA ($P = 0.001$; $r = 0.767$) and GSH/GSSG ($P < 0.001$; $r = 0.798$) all declined with the duration of lactation. These seems to be consistent with other studies which reported

the total antioxidant capacity and radical scavenging activity of breast milk in colostrum to be significantly higher than transitional and mature milks (Zarban, Taheri, Chahkandi, Sharifzadeh, & Khorashadizadeh, 2009; Yuksel, Yigit, & Cinar, 2015). There is some evidence that susceptible newborns are potentially vulnerable to oxidative stress due to the inefficiency of their antioxidant defense system or increase in free radical production (Yeung 2006). So neonates need more antioxidants from breast milk to protect their health. The results indicated the variations of antioxidant contents in breast milk could adapted to the demand degree of baby very well.

There was a rough correlation between the GSH/GSSG ratios and the contents of UA in human colostrums ($p < 0.05$, $r = 0.705$). But in mature milk, the correlation does not exist ($P > 0.05$).

In the study, we also found mothers who eat large vegetables and fruits have more AA in their breast milk than those who eat few or no vegetables and fruits ($P < 0.05$). But this pattern is not fitting to UA and GSH.

During the last few years, we have focused on the studies of the interactions of $[Ag(HIO_6)_2]^{5-}$ with bioactive and medically important molecules (Shi, 2009; Ma, Niu, Wang, Kang, & Shi, 2014; Ma, 2012; Ma, Fan, Xu, Kang, & Shi, 2011). We have found that $[Ag(HIO_6)_2]^{5-}$ can oxidize some reducible substances such as some soluble antioxidants and lead to CL signal enhancement remarkably for the $[Ag(HIO_6)_2]^{5-}$ -luminol system. We have thus used this property and established the HPLC- $[Ag(HIO_6)_2]^{5-}$ -luminol CL detection method, and realized simultaneously and sensitively determinations of AA, UA, GSH and GSSG in HBM.

Although AA, UA, GSH and GSSG all show electrochemical activity, the HPLC-ECD method for GSSG determinations exhibited higher detection limit (Monostori, Wittmann, Karg, & Túri, 2009) prohibiting the simultaneous determinations. The present method has not only realized simultaneous determination, but also has lower detection limits of these analytes. A comparison of HPLC in combination with the different detection methods for the determinations of AA, UA, GSH and GSSG is summarized in Table 4. Moreover, our proposed method in the present work is reliable, selective and sensitive.

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Vitae

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402 **Table.1.** Regression equation parameters of the four analytes.

403 **Table.2.** The method recovery for AA, GSH, UA and GSSG determination.

404 **Table.3.** The levels of AA, UA, GSH and GSSG in the healthy mothers' colostrum

405 and mature breast milk.

406 **Table.4.** Comparison of the detection limits of various HPLC methods for the

407 determination of AA, UA, GSH and GSSG.

408 **Fig.1.** Schematic diagram of the HPLC with post-column Ag(III)-luminol

409 chemiluminescence detection. 1: HPLC pump; 2: six-way injection valve; 3: HPLC

410 column; 4: CL reaction cell.

411 **Fig.2.** The typical chromatograms of the human milk samples and the sample spikings

412 with four analytes. Peaks: 1, AA; 2, GSH; 3, UA; 4, GSSG.

Table 1 Regression equation parameters of the four analytes.

Analytes	Linear range ($\mu\text{mol L}^{-1}$)	Linear equation	r	LOD (signal/noise=3 mol L^{-1})	RSD (n=7, %) ($5 \times 10^{-6} \text{ mol L}^{-1}$)
AA	0.5-20	$\Delta I = 1.304 \times 10^9 c - 116.4$	0.9912	4.60×10^{-8}	4.13
UA	0.1-20	$\Delta I = 0.9313 \times 10^9 c + 595.1$	0.9935	6.44×10^{-8}	3.12
GSH	0.25-10	$\Delta I = 1.546 \times 10^9 c + 420.9$	0.9954	4.85×10^{-8}	3.57
GSSG	0.1-10	$\Delta I = 1.562 \times 10^9 c + 412.85$	0.9937	3.88×10^{-8}	3.29

Table 2 The method recovery for AA, GSH, UA and GSSG determination.

	Recovery (%) (mol L^{-1}) (n=3)					
	2×10^{-6}	5×10^{-6}	1×10^{-5}	5×10^{-5}	1×10^{-4}	4×10^{-4}
GSH	-	98.73 ± 3.01	99.67 ± 1.18	93.33 ± 1.47	-	-
GSSG	96.90 ± 4.83	101.4 ± 4.36	100.2 ± 1.24	-	-	-
AA	-	-	-	106.7 ± 2.58	97.12 ± 5.41	91.80 ± 3.10
UA	-	-	-	96.10 ± 2.99	93.60 ± 2.75	92.16 ± 1.51

Table 3 The levels of AA, UA, GSH and GSSG in the healthy mothers' colostrum and mature breast milk.

	AA ($\mu\text{mol L}^{-1}$)	GSH ($\mu\text{mol L}^{-1}$)	UA ($\mu\text{mol L}^{-1}$)	GSSG ($\mu\text{mol L}^{-1}$)	GSH/GSSG
Colostrum (<7days, N=40)	298.3 ± 63.10	27.06 ± 12.17	127.3 ± 41.23	2.510 ± 1.58	14.44 ± 6.91
Mature milk (1-12months) (N=48)	193.4 ± 73.86	16.93 ± 11.86	76.10 ± 48.36	2.200 ± 1.11	8.330 ± 5.66
P	<0.05	<0.05	<0.05	>0.05	<0.05

Table 4 Comparison of the detection limits of various HPLC methods for the determination of AA, UA, GSH and GSSG.

Analytical method	sample	Detection limits ($\mu\text{mol L}^{-1}$)				References
		AA	UA	GSH	GSSG	
HPLC-FD	Human plasma			0.2	0.05	Ferin, Leonor Povao, & Baptista, 2012
HPLC-UV	Human seminal plasma	2.0	1.5	-	-	Kand'ár, Drábková, & Hampl, 2011
ion-exclusion HPLC-UV	Human plasma	0.306	0.428	-	-	Ferin, Leonor Pavão, & Baptista, 2013
HPCL-ECD	Human plasma	0.2	0.2	-	-	Rose, & Bode, 1995
HPCL-ECD	Human blood	-	-	0.011	0.156	Li, & Franke, 2009
HPCL-ECD	bladder tissue	0.5	0.01	0.05	-	Vovk, Bogataj, Rožskar, Kmetec, & Mrhar, 2005
HPLC-CL CL reagent: $[\text{Ag}(\text{HIO}_6)_2]^{5-}$ +luminol	Human milk	0.046	0.064	0.05	0.04	This method

Fig. 1. Schematic diagram of the HPLC with post-column Ag(III)-luminol chemiluminescence detection. 1: HPLC pump; 2: six-way injection valve; 3: HPLC column; 4: CL reaction cell.

Fig. 2. The typical chromatograms of the human milk samples and the sample spiking with four analytes. Peaks: 1, AA; 2, GSH; 3, UA; 4, GSSG.