Spectrophotometric determination of cobalt in horse urine using 2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]aniline as chromogenic reagent

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Abstract. Cobalt has been reported for being abused as an illegal doping agent due to its ability as an erythropoiesis-stimulating agent for enhancing performance in racehorses. Since 2015, cobalt is listed as a prohibited substance by the International Federation of Horseracing Authorities (IFHA) with a urinary threshold of 0.1 µg cobalt per mL urine. To prevent the misuse of cobalt in racehorse, a simple method for detection of cobalt is desirable. In this work, the detection of cobalt is based on the spectrometric detection of the complex formation between cobalt(II) and 2-(5-bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]aniline at pH 4. The absorbance of the complex is monitored at 602 nm. The metal:ligand ratio of the complex is 1:2. The calibration graph was linear in the range of 0 – 2.5 μM {Absorbance = (0.0825 ± 0.0013)[Co2+] + (0.0406 ± 0.0003), r² = 0.999} and the detection limit (3 SD of intercept)/slope was 0.044 μM. The proposed method has been successfully applied to horse urine samples with the recoveries in the range 91 – 98%.

1. Introduction
Since first observations of cobalt and its salts as an erythropoiesis-stimulating agent in 1929 [1], cobalt became an alternative agent to treat anemia [2, 3]. Cobalt stabilizes the hypoxia-inducible transcription factor (HIF) which activates erythropoietin (Epo) expression resulting in increase of red blood cells (RBCs) [4-6]. However, due to the adverse effects associated with this therapy, cobalt is no longer used for the treatment of anemia [4, 5, 7].

In view of cobalt’s ability as an erythropoiesis-stimulating agent, presumption about the illicit administration of cobalt as a performance-enhancer has been raised. The fact that cobalt salts can be orally taken and are affordable can lead to abuse of cobalt salt as an illegal doping agent [4, 5]. Recent reports have confirmed that cobalt is being abused in equine sports [5, 8]. Excessive administration of cobalt can be acutely toxic which can promote several health issues and even lead to death [8, 9]. In horse racing, cobalt is listed as a prohibited substance according to International Federation of...
Horseracing Authorities (IFHA) and the urinary threshold is 0.1 µg cobalt per mL urine or 1.7 µM [10]. For this reason, to prevent the misuse of cobalt in racehorse, simple detection of cobalt in urine is needed.

A standardized analytical methodology for the determination of cobalt in horse urine is not available [8]. Only a few papers reported methods for determination of cobalt either in horse urine or horse blood/serum samples [5, 6, 11]. According to Ho et al. [5], Knych et al.[6], and Popot et al.[11], the cobalt threshold in horse urine is based on total cobalt concentration which have been quantified by inductively coupled plasma – mass spectrometry (ICP-MS).

In general, determination of cobalt in various samples had been developed with mass-spectrometry-based detection, including ICP-MS, gas chromatography-mass spectrometry (GC-MS) [12], and electrospray ionization tandem mass spectrometry (ESI-MS-MS) [13]. Even though mass spectrometry-based method provides low limit of detection, they are complicated methods, high-cost, and require skill operator. Another common method which is widely used is spectrophotometry, which has been employed for the determination of cobalt(II) using 2-(5-Bromo-2-pyridylazo)-5-[(N-n-propyl-N-(3-sulfopropyl)amino)aniline (5-Br-PSAA) as a chromogenic reagent. Compared to mass spectrometry-based method, spectrophotometry is more convenient due to its lower cost and simplicity.

5-Br-PSAA (Figure 1) is a reagent which forms colored complex with metals including cobalt, nickel, iron, copper, and palladium [14, 15]. Cobalt forms complex with 5-Br-PSAA at the optimum pH range of 3 – 4.5 and with maximum absorption at 602 nm [14, 16]. According to Horiguchi et al., the molar absorptivity of this complex is 8.8 x 10^4 L mol^-1 cm^-1 at 602 nm, which is considered a high value [14]. In addition, 5-Br-PSAA is water-soluble reagent which is more convenient to use than other organic reagents. By using water-soluble reagent, the need for solvent extraction can be avoided [16]. Moreover, 5-Br-PSAA is commercially available at reasonable costs.

![Figure 1. Structure of 5-Br-PSAA [14].](image)

This paper describes the study of the determination of cobalt in horse urine using spectrophotometric method based on complex formation between cobalt(II) and 5-Br-PSAA as water-soluble reagent for simple, sensitive, and rapid analysis.

2. Experimental

2.1. Reagents

A stock standard solution of cobalt(II) was prepared by dissolving 0.1010 g of cobalt(II) chloride (97%) (Sigma-Aldrich, United States) in 25 mL of 2% of hydrochloric acid. The concentration of the stock cobalt solution was calculated from the equation  c = A/εb, where c is the concentration of the stock solution of cobalt (mol L^-1), A is the absorbance of the stock solution of cobalt, ε is the molar absorptivity (L mol^-1 cm^-1), and b is the path length of the cuvette (1.00 cm). The molar absorptivity was determined by measuring the absorbance at 509 nm of 0.017 M of a standard solution of cobalt(II) (standard for AA and ICP-OES, PerkinElmer, N9303766). The concentration of the stock cobalt solution was found to be 0.0175 M. Working solutions of cobalt were prepared by appropriate dilution of the standard solution of cobalt(II) with 0.05 M of acetate buffer pH 4.

A 0.001 M 5-Br-PSAA stock solution was prepared by dissolving 0.024 g of 2-(5-Bromo-2-pyridylazo)-5-[N-n-propyl-N-(3-sulfopropyl)amino]aniline, sodium salt (Dojindo Laboratories, Japan) in 50 mL of water.
A 0.042 M of acetic acid was prepared by diluting 1.21 mL of acetic acid glacial (Lab Scan, Thailand) to 500 mL of water. A 0.523 g of sodium acetate (Merck, Germany) was dissolved into 500 mL of acetic acid 0.042 M. These solutions were mixed to prepare a 0.05 M of acetate buffer solution pH 4.

2.2. Sample preparation
Horse urine samples were obtained from National Doping Control Centre, Mahidol University. The sample was first passed through a Sep-Pak C18 plus short cartridge (360 mg sorbent, 55-105 μm particle, Waters, Milford, United States) to remove coloured matrix. The cartridge was conditioned with methanol, followed by deionized water. A UV-visible spectrophotometer (Lambda 25, Perkin Elmer, United States) was used to measure absorbance of the complex at 602 nm.

3. Results and Discussions
The UV-visible absorption spectrum of the cobalt(II)–5-Br-PSAA complex was measured in the range of 300 – 700 nm at pH 4 (see Figure 2). The maximum absorption of free 5-Br-PSAA is at 457 nm. A maximum absorbance of the complex is observed at 602 nm where the free reagent has minimum absorbance.

![Figure 2. Absorption spectra of cobalt(II)–5-Br-PSAA complex.](image)

3.1. Job’s plot of cobalt(II)–5-Br-PSAA complex
The Job’s method was applied to determine the ratio between cobalt(II) (M) and 5-Br-PSAA (L).

\[ nM + pL \rightarrow M_nL_p \]  \hspace{1cm} (1)

A series of solution, where the total of mole M and mole L is constant, were prepared. Therefore, the total concentration of cobalt(II) and 5-Br-PSAA was also constant when the ratio was continuously varied. The maximum absorbance of \( M_nL_p \) complex corresponds to the maximum concentration of \( M_nL_p \) complex [17]. Thus,

\[ \frac{n}{p} = \frac{X_M}{X_L} \]  \hspace{1cm} (2)

Where, \( X_M \) is mole fraction of M and \( X_L \) is mole fraction of L.
The results in Figure 3 show that the maximum absorbance is observed when the mole fraction of ligand $X_L$ is 0.67 and $X_M$ is 0.33 (Figure 3). Therefore, the composition of complexes obtained is Co(II) : 5-Br-PSAA = 1 : 2. The result was in good agreement with the previous work of Iburaim et al.[18].

3.2. Calibration and detection limit

Calibration graph for cobalt is linear (Figure 4) for 0 – 2.5 μM ($r^2 = 0.999$), with the equation:

$$\text{Absorbance (602 nm)} = (0.0825 \pm 0.0013) [\text{Co}^{2+}] + (0.0406 \pm 0.0003)$$

Limit of detection (LOD) of cobalt solution was calculated from [19]

$$\text{LOD} = \text{intercept} + 3 \, S_{\text{y/x}}$$

LOD was 0.044 μM. In this work the urine sample from the Sep-Pak clean-up was diluted 10 fold with the acetate buffer. This method is suitable for analysis of cobalt in horse sample because urinary cobalt threshold after dilution is 0.17 μM (0.01 μg/mL).

Figure 3. Modified job's plot after baseline subtraction.
Conditions: Co(II) + 5-Br-PSAA = 5 μM; acetate buffer 0.05 pH 4.

Figure 4. Calibration graph of Co(II) (0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 μM) obtained at 602 nm.
Conditions: 5-Br-PSAA 50 μM, acetate buffer 0.05 M pH 4.
3.3. Interference study (nickel and zinc)
Recent reports found a significant amount of undeclared nickel in performance enhancing products [20,21]. Thevis et al. in their pilot study reported that the mean urinary nickel level was 0.1 (± 0.086) μM from 200 equine doping control urine samples. Therefore, the interference of nickel(II) was studied since nickel(II) also form a complex with 5-Br-PSAA.

Besides, the interference of zinc was also studied. Although zinc(II) does not form coloured complex with 5-Br-PSAA, it may affect the quantitation of the cobalt-complex when the urinary zinc excretion is high. Normally, the level of urinary zinc excretion in horse does not change with excess of zinc dietary intake [22].

The results are summarized in Table 1. Both nickel(II) and zinc(II) does not significantly interfere at levels up to 1.7 μM.

| Added (µM)       | Found (µM)       | Recovery (%) |
|------------------|------------------|--------------|
| Cobalt(II) 1.7 μM| Nickel(II) 1.7 μM| 1.716 ± 0.003| 101          |
| Cobalt(II) 1.7 μM| Zinc(II) 1.7 μM  | 1.674 ± 0.001| 98           |

Table 1. Interference of nickel(II) and zinc(II) on the determination of 1.7 μM cobalt(II).

Conditions: 5-Br-PSAA 50 μM, acetate buffer 0.05 M pH 4, absorption at 602 nm.

3.4. Application
The method was applied to the determination of Co(II) in horse urine samples. After sample pre-treatment using the C18 cartridge, 1.7 μM Co(II) were spiked into the diluted horse urine samples. Table 2 shows that the recoveries ranged between 91 – 98%.

| Horse urine sample | Concentration of diluted sample (µM)b | Added (µM) | Found (µM)b | Recovery (%) |
|--------------------|--------------------------------------|------------|-------------|--------------|
| AA                 | Not detected                          | 1.7        | 1.663 ± 0.023| 98           |
| AI                 | 0.107 ± 0.011                         | 1.7        | 1.647 ± 0.016| 91           |
| AW                 | 0.058 ± 0.002                         | 1.7        | 1.640 ± 0.056| 93           |
| AT                 | Not detected                          | 1.7        | 1.596 ± 0.036| 94           |
| AO                 | Not detected                          | 1.7        | 1.572 ± 0.016| 92           |

Table 2. Recovery study of cobalt determination in horse urine samples.

Conditions: 5-Br-PSAA 50 μM, acetate buffer 0.05 M pH 4, absorption at 602 nm.
b The sample was diluted ten fold
b Mean of concentration ± SD, n = 3

4. Conclusions
In the present work, a simple, convenient, and rapid spectrophotometric method have been successfully developed for the determination of cobalt using 5-Br-PSAA as water-soluble reagent. This method is sensitive and suitable to be applied to horse urine. Therefore, we propose that this spectrophotometric method can be an alternative method for screening of cobalt in equine sport.

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