A Rapid and Effective HPLC Method for Identification of *Bovis Calculus* in Chinese Patent Medicines

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Abstract: **Objective** Due to the limited resource and the large demand, many kinds of *Bovis Calculus* (BC) including artificial *Bovis Calculus* (ABC), *in vivo* cultured *Bovis Calculus* (*in vivo* CBC), and *in vitro* cultured *Bovis Calculus* (*in vitro* CBC) were used in Chinese patent medicines (CPMs). Previous studies have shown that the chemical constituents of ABC and their properties were different from other BC. The two types of CBC with much higher price than ABC were approximately equivalent with natural *Bovis Calculus* in quality and clinical effect. The aim of the study is to establish a rapid and effective method for the identification of BC in CPMs. **Methods** An HPLC method with the higher specificity for analyzing bilirubin was established to distinguish ABC from other three kinds of BC by comparing the change of bilirubin content with the addition of EDTA-2Na as the extraction solvent and stabilizer. **Results** The bilirubin content in CPMs containing ABC was basically unchanged, while that in CPMs containing other kinds of BC showed significant difference. The proposed method was employed to analyze a variety of CPMs containing *Bovis Calculus* (CPMBCs) and proven to be universal. **Conclusion** An effective analytical method is established for the quality control of CPMBCs and further ensures the safety and efficacy of these drugs in clinical practice.

Key words: artificial *Bovis Calculus*; bilirubin; *Bovis Calculus*; Chinese patent medicines; cultured *Bovis Calculus*; EDTA-2Na solution

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Introduction

Bovis Calculus (BC, *Niuhuang* in Chinese), usually known as natural *Bovis Calculus* (NBC), is the dry gallstone of *Bostaurus domesticus* Gmelin. It is a precious Chinese materia medica (CMM) widely used in the clinics for restoring consciousness by reducing fire and eliminating phlegm, reliving convulsion, and counteracting toxicity according to the theory of traditional Chinese medicine (TCM) which was practical in Asian area for thousands of years (Pharmacopoeia Committee of P. R. China, 2010). Modern pharmacological studies have shown that BC has the function of sedation, antihyperspasmia, relieving fever, analgesia, and diminishing inflammation (Cai *et al*, 2004; Wang, 2002; Yasukawa, Iida, and Fujimoto, 2009; Mizuno *et al*, 2005; Takahashi, 2009). There are about 500 Chinese patent medicines containing BC (CPMBCs) in China. Due to the limited resource and the large demand for NBC, artificial *Bovis Calculus* (ABC), *in vivo* cultured *Bovis Calculus* (*in vivo* CBC), and *in vitro* cultured *Bovis Calculus* (*in vitro* CBC) were successively approved as the substitutes for NBC in

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order to increase its supply for pharmaceutical industries.

Previous studies have shown that the chemical constituents of ABC and their properties are different from those of NBC, *in vitro* CBC, and *in vivo* CBC (Zhang, 1995; Cai, Qiu, and Liu, 2004; Ding *et al*, 2004; Zhao and Ruan, 2007). Types of CBC with much higher price than ABC are approximately equivalent with NBC in quality and clinical effect. With regards to this, under the *State Food and Drug Administration of China Guidelines* that medicinal preparations containing BC for acute and severe diseases in National Drug Standards and new drugs containing BC approved by Pharmaceutical Supervisory and Administrative Department could only use NBC or its substitutes (referring to *in vivo* CBC and *in vitro* CBC), banning the replacement using ABC. However, due to the lack of accurate analysis technique, most of the methods currently used are not able to identify the exact type of BC, which may result in confusion in selecting material and great quality difference for CPMBCs.

In order to accurately monitor the quality of CPMBCs, firstly, the type of BC in the prescriptions should be acknowledged, i.e, whether ABC or other three kinds of BC (NBC, *in vitro* CBC, and *in vivo* CBC) were used. Based on the literatures, the major bioactive ingredients of BC are cholic acid and bilirubin (Zhao and Ruan, 2007). NBC, *in vitro* CBC, and *in vivo* CBC contain cholic acid and deoxycholic acid, while ABC contains hyodeoxycholic acid (HDCA) which is a unique component to distinguish ABC from others. However, some ABC without adding HDCA was easily mistaken for other three kinds of BC in routine inspection tests. Therefore, the identification of HDCA by thin layer chromatography (TLC) was only a supplement means for judging the type of BC.

Taking into account the great difference of bilirubin content between ABC and others, the determination of bilirubin warrants further investigation on the novel method to identify the type of BC within a drug. Nowadays, 45 CPMBCs were recorded in *Chinese Pharmacopoeia 2005* (volume I), among which 29 preparations were carried out with TLC and one with chemical reaction of furfurol for the identification of cholic acid, only one preparation applied the thin layer chromatography scanning method (TLCS) for the determination of cholic acid. None of the preparation includes bilirubin component test in all of these CPMBCs. Hence, it is necessary to establish an efficient and simple method for the determination of bilirubin in CPMBCs.

Bilirubin is a linear tetrapyrrole derivative with a spatial structure and optical activity (Fig. 1). There are many isomers of bilirubin, and bilirubin IXα often exists in living organisms. In the physiological pH conditions, bilirubin IXα has the sheet secondary structure formed by the intra-molecular hydrogen bond (Zheng, Li, and Feng, 1996) (Fig. 1). Due to the intra-molecular hydrogen bond, bilirubin is insoluble in water, but soluble in dichloromethane and other organic solvents. The intra-molecular hydrogen bond could easily be destroyed by light, heat, and alkaline conditions (Bonnett, Davies, and Hursthouse, 1976), etc. On the other hand, bilirubin could easily combine with metal ions and be oxidized to biliverdin (Zheng, Yang, and Zeng, 2000). Bilirubin could combine with glucuronic acid or taurine by hydrogen bonds to form conjugated bilirubin, it could also combine with protein by covalent bonds to form covalent bilirubin. ABC mostly contains free bilirubin, while the other three kinds of BC (NBC, *in vivo* CBC, and *in vitro* CBC) mostly contain up to 90% conjugated and covalent bilirubin. Moreover, bilirubin content in ABC is extremely lower than that in the other three kinds of BC (Zhang, 2003).

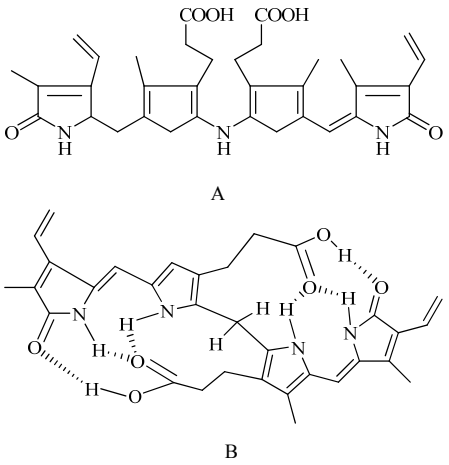

Fig. 1 Structures of bilirubin IXα

A: plain structure B: structure for its hydrogen bond network

In the past, the determination of bilirubin in BC was mostly carried out by HPLC (Yang, Zhen, and Bai, 2004; Yan *et al*, 2007; Guo, Su, and Xue, 2008; Yang, Huang, and Li, 1995; Liu *et al*, 2000; Fang *et al*, 2002; Liang *et al*, 2006; Wu and Xie, 1995) and UV (Wu *et al*, 1996; Li *et al*, 2005). However, these two methods both have drawbacks. The method of UV was inaccurate which was affected by the quantity of the ingredients in CPMs resulting in interference. In the literature-reported HPLC method, bilirubin was unstable in the mobile phase, and easily degraded in the test solution, even it could not be completely extracted by extraction solution.

Therefore, a novel approach with high specificity for bilirubin determination in CPMBCs was developed by HPLC in this study. On the basis of optimizing mobile phase and extraction solvent, EDTA-2Na solution was introduced to improve the stability and the extraction efficiency. By observing the change of bilirubin content with the addition of EDTA-2Na solution, the established method could not only effectively distinguish ABC from NBC, *in vitro* CBC, and *in vivo* CBC, but also accurately determine bilirubin to ensure the quality of preparations. By validating on the multiple kinds of CPMBCs, the method established in this study was proved to be universal and has great application value.

Materials and methods

Chemicals and materials

Bilirubin reference substance was purchased from National Institutes for Food and Drug Control (Beijing, China), and the purity of the compound was higher than 98%. Six kinds of CPMBCs samples (a total of 31 batches) were provided by different manufactures in China. They are Niuhuang Baolong Tablets (NBT), Qiwei Xinxiao Pills (QXP), Jufang Zhibao Powders (JZP), Angong Niuhuang Pills (ANP), Angong Niuhuang Powders (ANPO), and Niuhuang Baolong Pills (NBP), respectively. Details of the sample source are listed in Table 1. HPLC grade acetonitrile was from Merck (Darmstadt, Germany). Dichlormethane, methanol, acetic ether, 36% acetic acid, and ethylene diamine tetraacetic acid disodium salt (EDTA-2Na) were of analytical grade obtained from Shanghai Chemical Agent Company, China National Pharmaceutical Group (China). Deionized water (18 MΩ) was prepared by distilled water through a Milli-Q system (USA).

Due to the long production cycle and high price, *in vivo* CBC is currently rare on the market. Thus, CPMs containing *in vivo* CBC are not included in this study. *In vitro* CBC was produced on a large scale, the quality is relatively stable, and the efficacy is close to NBC. Therefore, in this study, *in vitro* CBC was investigated as the substitute of NBC.

Preparation of reference substance solutions

Stock reference substance solution of bilirubin was prepared in dichloromethane, and the concentration was 61.0 μg/mL. The working solutions were obtained by diluting the stock solution with dichloromethane to a series of proper concentration, such as 1.22, 2.44, 4.88, 12.2, 24.4, 48.8, and 61.0 μg/mL, respectively. All these solutions were stored at 4 ℃ until use.

Preparation of sample solutions and negative sample solutions

An appropriate amount of samples was ground and weighed accurately in a brown volumetric flask, the amount of EDTA-2Na solution (0.2 mol/L) was added accurately (When preparing, to heat moderately to make it dissolve completely, to allow to cool before use, and to prepare it before use), and mixed well. Then, an appropriate amount of water-saturated CH_2Cl_2 was added accurately and then weighed. The samples were extracted by ultrasonication for 20 min in an ice bath (power of 180 W and frequency of 42 kHz). After ultrasonication, the samples were weighed again and the loss of weight was replenished with dichloro- methane, stirred well, and centrifuged. The lower solutions (containing dichloromethane) were filtered through 0.22 μm membrane filters, and the successive filtrate was used as the test solution. The negative sample solutions were prepared according to the above method.

HPLC conditions

The HPLC analysis was carried out using an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, a microdegasser, an autoplate-sampler, a thermostatically controlled column apartment, and a PDA detector. The chroma-tography separation was performed on a Kromasil-C_{18} column (150 mm× 4.6 mm, 5 μm; Eka Nobel) at a temperature of 30 ℃. Acetonitrile and 1% acetic acid

Table 1 Samples of CPMBCs, negative samples, and quantitative analytical results of 31 CPMBCs samples ($\overline{X} \pm s$, $n = 3$)

Sample No.	Sample names	Sample code[a]	Types of BC in samples	Batch No.	Negative samples	Bilirubin contents / ($mg \cdot g^{-1}$)
	NBT	LYS	ABC	061001	Y	0.075 ± 0.002
1				061002		0.074 ± 0.001
				061003		0.078 ± 0.002
	QXP	LYS	ABC	060401	Y	0.229 ± 0.002
2				060402		0.240 ± 0.003
				060403		0.238 ± 0.002
	JZP	FLX	*in vitro* CBC	080604	Y	12.400 ± 0.058
3				080605		11.900 ± 0.088
				080606		11.700 ± 0.071
	ANP	WHDP	ABC	SR[b] 4-1	Y	0.277 ± 0.006
		WHDP	*in vitro* CBC	SR4-2	Y	11.440 ± 0.081
		DD	*in vitro* CBC	050901	N	8.533 ± 0.075
		SYT	NBC	0801103	N	1.567 ± 0.028
		XZ	*in vitro* CBC	20080401	N	12.233 ± 0.088
4				20080402		11.833 ± 0.073
				20080403		13.067 ± 0.069
		TRT	NBC	9017003	N	9.633 ± 0.065
				8017016		8.167 ± 0.070
				8017015		8.200 ± 0.083
	ANPO	WHDP	ABC	SR5-1	Y	1.862 ± 0.024
		WHDP	*in vitro* CBC	SR5-2	Y	47.172 ± 0.307
5		LYS	ABC	20081201	N	0.530 ± 0.009
				20081202		0.530 ± 0.008
				20081203		0.500 ± 0.008
	NBP	WHDP	ABC	SR6-1	Y	0.093 ± 0.002
		WHDP	*in vitro* CBC	SR6-2	Y	5.890 ± 0.078
		YZ	ABC	20080401	N	0.021 ± 0.001
6				20080402		0.023 ± 0.001
				20080403		0.019 ± 0.001
		TRT	ABC	9015066	N	0.017 ± 0.001
				6015047		0.020 ± 0.001

[a] Represent samples of CPMBCs and negative samples from eight different manufacturers (LYS, FLX, WHDP, DD, SYT, XZ, TRT, and YZ)

[b] Refer to samples for research

Y: Note that the manufacturer supplied the corresponding negative sample

N: Note that the manufacturer did not supplied the corresponding negative sample

(95:5) were used as the mobile phases. The flow rate was set at 1.0 mL/min, the injection volume was 5 μL, and the peaks were detected at 450 nm.

Results and discussion

Selection of mobile phase

In the literatures, the mixed solution of chloroform-methanol-hydrochloric acid, dimethyl sulfoxide, or *N*, *N*-dimethylformamide was mostly used as the mobile phase for analysis of bilirubin in BC (Guo, Su, and Xue, 2008; Fu, Gao, and Chen, 2006; Chen *et al*, 2004; Jiang *et al*, 2004; Li and Yang, 2008). However, bilirubin was easily decomposed in the above-mentioned mobile phase. It is due to the reason that the mobile phase with strong acidity or polarity could easily result in the decomposition of bilirubin peak. Therefore, the following weakly acidic mobile phases were investigated (I) methanol-acetonitrile-1% glacial acetic acid solution:

(88:10:2); (II) acetonitrile-1% glacial acetic acid solution (87:13); (III) acetonitrile-1% glacial acetic acid solution (95:5). The results showed that the bilirubin peak still decomposed in the mobile phase I, but remained stable in the mobile phases II and III within 12 h. Taking into account of the retention time, theoretical plate number, etc., the mobile phase III was finally selected as the suitable mobile phase for bilirubin analysis in this study. The chromatographic parameters of bilirubin for each mobile phase were listed in Table 2.

Table 2 Chromatographic parameters of bilirubin for each mobile phase tested

Mobile phases	t_R / min	Peak areas per gram of samples	Theoretical plate numbers
I	9.654	305.72	1055
II	8.792	302.83	2557
III	8.549	285.23	3154

Choice of extraction solvent for sample solutions

The solubilities of free, conjugated, and covalent bilirubin are different. In order to accurately determine bilirubin, it is important to investigate the extraction solvents to obtain all three types of bilirubin.

In the literature, ABC was generally extracted by non-acidic solvent, such as dichloromethane, etc. Acid or polar solvent could make conjugated bilirubin and covalent bilirubin free, and was often used for NBC, *in vitro* and *in vivo* CBC (Guo, Su, and Xue, 2008). Therefore, seven commonly used extraction solvents reported in literature were investigated (Guo, Su, and Xue, 2008; Fu, Gao, and Chen, 2006; Chen *et al*, 2004; Jiang *et al*, 2004; Li and Yang, 2008; Xu *et al*, 2004; Zhang, Dong, and Liu, 2005; Xu, Wen, and Chen, 2005), namely (1) chloroform-methanol-hydrochloric acid-water (90:10:0.015:0.3), (2) DMSO-acetonitrile (9:4), (3) chloroform-glacial acetic acid (4:1), (4) chloroform-methanol-water (90:10:0.3), (5) chloroform-methanol (90:10), (6) chloroform, and (7) dichloromethane. The chromatographic parameters of bilirubin for each extraction solvent were listed in Table 3.

Our results showed that the bilirubin peak easily decomposed into multiple peaks when using the No. 1—3 solvents. This may be due to the CPMs mentioned in the literature containing fewer species of CMM in addition to BC, and the interference to the bilirubin analysis is small in the reported settings. However, the CPMs tested in our study contain mineral drugs, such as cinnabar, realgar, etc, which may result in the interference of metal ions which could easily form complexes with bilirubin in solution. Thus, the changes in the chromatographic profiles of bilirubin were caused. When using the solvents No. 4—7, the single peak of bilirubin was obtained. However, the peak area in samples containing NBC or *in vitro* CBC was much smaller than that obtained by using the solvents of No. 1—3. It may indicate that the bilirubin in those samples was not completely extracted, probably because the solvents No. 4—7 could not make conjugated or covalent bilirubin free. Therefore, the key of accurate determination of bilirubin in the samples containing NBC or *in vitro* CBC is to make conjugated or covalent bilirubin completely free and ensure the stability of the extraction process.

Table 3 Chromatographic parameters of bilirubin for each extraction solvent tested

Extraction solvents	t_R / min	Peak areas per gram of sample
1	10.201	903.16
2	9.578	977.74
3	10.197	924.51
4	9.856	28.47
5	10.078	27.64
6	10.245	28.22
7	9.631	29.86

Choice of extraction solvent

As mentioned by two studies that a little amount of ethylene diamine tetraacetic acid (EDTA) could help prevent the oxidation of bilirubin (Fog and Bugge-Asperheim, 1964; Li *et al*, 1990), since EDTA acts as a chelating agent with the ability to "sequester" the metal ions, metal ions may exhibit the diminished reactivity in extraction solvent after being bound by EDTA. Among the available EDTA salts, EDTA-2Na which has good solubility in water (10.8 g/100 g at 22 ℃, equivalent to the concentration of about 0.29 mol/L) was chosen in this study. Taking into account that the conjugated and covalent bilirubin is soluble in water, therefore, 0.2 mol/L EDTA-2Na solution was added to the three kinds of commonly used extraction solvents

respectively, namely water-saturated CH_2Cl_2, water-saturated $CHCl_3$, and water-saturated ethyl acetate.

Six copies of each following sample were weighed respectively, namely NBT 0.8 g, QXP 0.2 g, JZP 0.05 g, ANP-1 (research sample containing ABC) 0.23 g, ANP-2 (research sample containing *in vitro* CBC) 0.05 g, ANPO-1 (research sample containing ABC) 0.13 g, ANPO-2 (research sample containing *in vitro* CBC) 0.03 g, NBP-1 (research sample containing ABC) 0.5 g, and NBP-2 (research sample containing *in vitro* CBC) 0.25 g. We conducted the investigations in accordance with the following six kinds of sample preparation methods: 1) water-saturated CH_2Cl_2 (20 mL) was added accurately; 2) 0.2 mol/L EDTA-2Na solution (2 mL) was added accurately, mixed well, then 20 mL of water-saturated CH_2Cl_2 (20 mL) was added accurately; 3) water-saturated $CHCl_3$ was added accurately; 4) 0.2 mol/L EDTA-2Na solution (2 mL) was added accurately, mixed well, then water-saturated $CHCl_3$ (20 mL) was added accurately; 5) water-saturated ethyl acetate (20 mL) was added accurately; 6) 0.2 mol/L EDTA-2Na solution (2 mL) was added accurately, mixed well, then water-saturated ethyl acetate (20 mL) was added accurately. The above obtained sample solutions were weighed respectively and extracted by ultrasonication for 20 min in an ice bath (power of 180 W and frequency of 42 kHz), then the test solutions were prepared in accordance with the above mentioned methods. An aliquot of 5 μL of each test solution was injected accurately into the column, respectively. Peak areas were recorded and calculated. The results were shown in Table 4.

Table 4 Peak areas of bilirubin in representative samples obtained by different extraction methods ($\bar{X} \pm s$, $n = 3$)

Sample names (Batch No.)	Peak areas per gram of samples					
	1	2	3	4	5	6
NBT (061001)	208.76 ± 2.40	160.24 ± 1.76	186.32 ± 1.53	171.04 ± 1.20	140.42 ± 2.33	60.37 ± 0.88
QXP (060401)	577.68 ± 2.96	562.53 ± 2.03	501.60 ± 2.89	598.10 ± 2.89	395.58 ± 2.96	370.63 ± 2.08
JZP (080604)	437.55 ± 2.03	16 916.37 ± 3.84	320.46 ± 2.65	16 758.71 ± 3.46	55.75 ± 0.67	13 652.55 ± 3.79
ANP-1 (SR4-1)	437.23 ± 1.76	771.28 ± 2.60	528.32 ± 2.31	370.53 ± 1.45	170.64 ± 1.76	257.91 ± 2.89
ANP-2 (SR4-2)	0.00	3020.64 ± 2.85	0.00	3024.29 ± 3.18	0.00	2681.85 ± 3.48
ANPO-1 (SR5-1)	7088.87 ± 3.21	8602.33 ± 3.61	7047.87 ± 2.65	8590.10 ± 3.28	1794.62 ± 2.91	928.45 ± 1.76
ANPO-2 (SR5-2)	0.00	26 624.48 ± 3.79	0.00	27 961.38 ± 3.76	0.00	23 398.75 ± 3.28
NBP-1 (SR6-1)	61.68 ± 0.88	77.92 ± 1.15	55.47 ± 0.88	75.02 ± 1.20	16.92 ± 0.33	25.76 ± 0.58
NBP-2 (SR6-2)	174.21 ± 1.76	1611.24 ± 2.31	179.52 ± 2.03	1608.28 ± 2.65	0.00	918.09 ± 2.31

By adding EDTA-2Na solution, the extraction efficiency of bilirubin in CPMs containing *in vitro* CBC was significantly increased, while there was few change observed in bilirubin level of CPMs containing ABC (Table 4). On the other hand, the extraction efficiency of water-saturated CH_2Cl_2 was slightly better than that of water-saturated $CHCl_3$, and that of water-saturated ethyl acetate was relatively poor. EDTA-2Na solution could not only reduce the bonding force between bilirubin and its conjugate, make bilirubin free, but also combine with metal ions to form stable coordination compounds, exclude the interference of metal ions in the extraction process so as to prevent the oxidation of bilirubin, and ensure the stability of bilirubin. As a result, dichloromethane and proper amount of EDTA-2Na solution were selected as extraction solvent.

In the literature, the extraction solvent of bilirubin in CPMs containing ABC or *in vitro* CBC was often added with acid in order to make bilirubin free and ensure the extraction of bilirubin completely. In order to investigate whether the bilirubin could be extracted completely by adding EDTA-2Na solution, the representative CPMs (JZP) and *in vitro* CBC were selected for investigation and extracted with CH_2Cl_2-EDTA-2Na, acid-CH_2Cl_2, and acid-CH_2Cl_2-EDTA-2Na, respectively. Here, water-saturated CH_2Cl_2, EDTA-2Na solution (0.2 mol/L), hydrochloric acid (0.24%), phosphoric acid (0.24%), and glacial acetic acid (0.24%) were used. The peak areas of bilirubin for each extraction solvent were listed in Table 5. The results showed that by adding sufficient EDTA-2Na solution, for JZP, extraction efficiencies of CH_2Cl_2-

EDTA-2Na and acid-CH_2Cl_2-EDTA-2Na were roughly the same, and much higher than that of acid-CH_2Cl_2; for *in vitro* CBC, the extraction efficiencies of the three systems were basically the same. It could be seen that the metal ions had a greater impact on the extraction of bilirubin, EDTA-2Na solution could effectively remove the interference to guarantee the stability of bilirubin, and the extraction solvent of CH_2Cl_2-EDTA-2Na without acid could also extract bilirubin completely.

Table 5 Peak area per gram of bilirubin for each extraction solvent tested

Extraction solvents	Peak areas per gram	
	JZP	*In vitro* CBC
CH_2Cl_2-EDTA-2Na	5134.77	65 337.29
HCl-CH_2Cl_2	3762.38	46 812.52
H_3PO_4-CH_2Cl_2	1622.46	25 495.24
CH_3COOH-CH_2Cl_2	1244.62	10 764.87
HCl-CH_2Cl_2-EDTA-2Na	5207.86	65 736.35
H_3PO_4-CH_2Cl_2-EDTA-2Na	5124.60	65 003.14
CH_3COOH-CH_2Cl_2-EDTA-2Na	5180.46	65 705.60

Choice of extraction time

As bilirubin is unstable to light and heat, ultrasonication in an ice bath (power of 180 W and frequence of 42 kHz) was chosen to extract the samples away from light. Ultrasonic time was investigated for 10, 20, 30, and 40 min. The results showed that in a variety of preparations, bilirubin had been completely extracted after ultrasonication for 20 min. Thus, ultrasonication for 20 min in an ice bath was used to prepare the samples.

Identification of types of BC in CPMBCs

The following six batches of research samples were selected for test, ANP (SR4-1 and SR4-2), ANPO (SR5-1 and SR5-2), and NBP (SR6-1 and SR6-2). A proper amount of each sample was ground to fine powder, and weighed accurately in a brown volumetric flask, a certain amount of 0.2 mol/L EDTA-2Na solution (0, 1, 2, 3, 4, and 5 mL) was added, respectively, then the test solution was prepared in accordance with the above mentioned methods. The results were shown in Fig. 2.

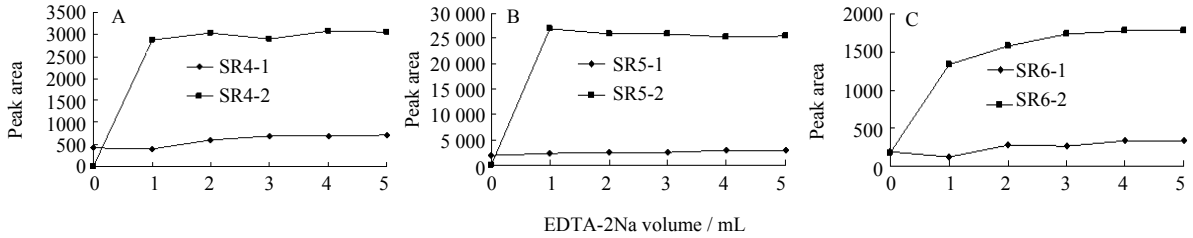

Fig. 2 Extraction efficiency (bilirubin content) in six research samples with adding of EDTA-2Na solution

A: ANPI (SR4-1 and SR4-2); B: ANPO (SR5-1 and SR5-2); C: NBP (SR6-1 and SR6-2)

As seen from Fig. 2, the bilirubin content of CPMs containing *in vitro* CBC was rapidly increased with adding EDTA-2Na solution gradually, while that of CPMs containing ABC showed no significant change. As the bilirubin content and its existing form were almost the same in NBC, *in vivo* CBC, and *in vitro* CBC, the difference could be used to identify the type of BC. For example, by observing the change of bilirubin level after adding a certain amount of EDTA-2Na solution to an unknown sample containing BC, the types of BC (ABC or other three species of BC) could be discriminated. Thus the method will contribute to detect whether the sample contains ABC, and eliminate the confusion and misuse of different kinds of BC. The results further showed that bilirubin could be completely extracted in the selected samples by adding 5 mL of 0.2 mol/L EDTA-2Na solution.

Method validation

Specificity The specificity was demonstrated by observing chromatograms of the real samples and the negative samples which did not contain BC. There was no interference with the analyte peaks in the chromatograms of real samples, and the chromatograms of the negative samples did not exist the corresponding peak of bilirubin. These results showed good specificity of the method for analysis.

Linearity, limit of detection and limit of quantification Dichloromethane stock solutions containing reference compound were diluted to appropriate concentration for the construction of

calibration curves. A set of the solutions at different concentration were analyzed in duplicates, and then the calibration curves were constructed by plotting the peak areas versus the amount of bilirubin injected onto the column. The calibration curve for the determination of bilirubin (Y = 5.0486X + 4.6267) was linear over the range of 0.0061—0.305 μg. The correlation coefficient (r^2) obtained was 0.9999. The limit of detection (LOD) and limit of quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The LOD and LOQ were lower to 0.002 and 0.0057 μg for bilirubin, respectively.

Precision, repeatability, and stability Intra- and inter-day variations were chosen to determine the precision of the developed assay. For intra-day variability test, the standard solution was analyzed for six replicates within 1 d, while for inter-day variability test, the solution was examined in duplicates for consecutive 3 d. Variations were expressed by the relative standard deviations (RSD) for intra- and inter-day, which were less than 0.18% and 3.12%, respectively. The repeatability of the developed method was evaluated at three levels (high, middle, and low) of each sample. The samples of each level were prepared triplicates and analyzed as mentioned above. The repeatability present as the average RSD of three kinds of concentration was ranged from 0.18% to 3.87%. The stability of bilirubin was tested with the reference substance solution and samples were prepared from each CPMBC sample at room temperature (25 ℃) and were analyzed every 2 h for 26 h. The data expressed as RSD values were 0.54% for reference substance solution, and ranged from 0.46% to 4.36% for CPMBCs samples. The results showed that bilirubin was stable in 26 h. Consequently, the stability was greatly improved because of adding EDTA-2Na, compared with those reported previously by other sample preparation methods. In the literature, bilirubin would be stable in 4—10 h, and the analysis was best completed as soon as possible (Guo, Su, and Xue, 2008; Deng *et al*, 2007; Fu *et al*, 2006; Hu *et al*, 2005).

Recovery The recovery of the method was investigated employing the standard addition method. Bilirubin was added at three different levels (approximately equivalent to 0.8, 1.0, and 1.2 times of the concentration of the matrix) with three parallels at each level. The samples were extracted and analyzed using the method mentioned above. The average recoveries in three spiked levels were ranged from 95.0% to 105.0% with RSDs less than 5%. The results showed the acceptable losses in preparation procedure of CPMBCs.

As described above, the data demonstrated that the analytical method was suitable for the quantitative analysis of bilirubin in different CPMBCs.

Application to samples

The validated HPLC method was applied to analysis of bilirubin in 31 commercial samples of CPMBCs, i.e., three tablets, twenty pills, and eight powders. The typical HPLC profiles of bilirubin and one sample (JZP 080604) were shown in Fig. 3, and the bilirubin contents of 31 samples were listed in Table 1. The results showed that the contents of bilirubin had no significant difference among the same kind of commercial samples containing the same kind of BC. However, in the same type of samples for research, there is a significant difference between samples containing ABC and those containing *in vitro* CBC. Compared with the samples containing ABC, the bilirubin contents in the samples containing *in vitro* CBC were 40 — 60 times more. Therefore, it is scientific and necessary to distinguish the species of BC in CPMBCs by determining bilirubin.

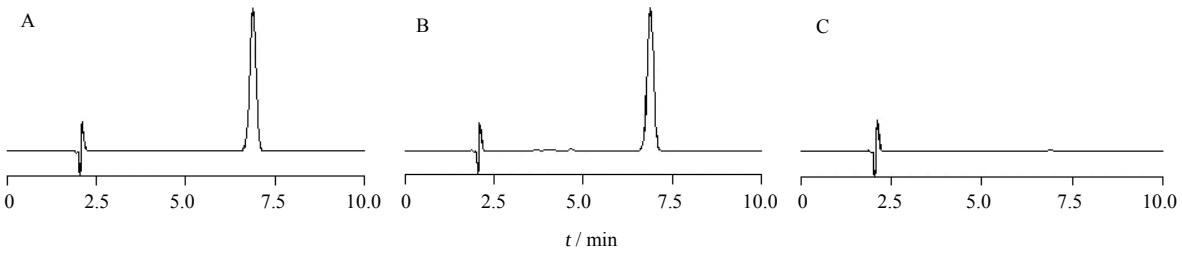

Fig. 3 Typical HPLC chromatograms of bilirubin (A), one sample solution (JZP 080604, B), and negative sample solution (JZP 080604, C)

Conclusion

A simple and effective HPLC method was established for distinguishing ABC from other types of BCs. The type of BCs could be discriminated by comparing the change of bilirubin content after adding EDTA-2Na solution, that is, the bilirubin content of preparations containing ABC is basically the same, while that of preparations containing other types of BC shows the significant changes. Differing from the traditional extraction method using acid, the proposed method could shield the metal ions effectively, and determine bilirubin precisely. Firstly, as the extraction solvent and stabilizing agent, EDTA-2Na could combine with conjugate (e.g., glucuronic acid) more easily than bilirubin, thus making bilirubin free. Secondly, EDTA-2Na could combine with the metal ions or some oxides so as to prevent the oxidation of bilirubin. Thirdly, the pH value of 0.2 mol/L EDTA-2Na solution ranges between 4 to 5, and this weak acidic environment will help maintain the stability of intramolecular hydrogen bond of bilirubin. The demonstrated tests showed that the bilirubin contents of all prepared sample solutions remained stable up to 26 h, which solved the stability problem of bilirubin in the process of extraction and determination.

The proposed method for the determination of bilirubin is applicable to a number of CPMBCs, the difference only lies in the sample and reagent volume. From the results of this study it could be seen that the analytic technique of BC could be greatly improved, which could help to distinguish the types of BC and make big leaps of progress in the quality control of CPMBCs possible with further investigations.

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