技术与方法

一种安全高效的血红蛋白纯化方法*

臧家涛 刘建仓 徐 竞 李东红 刘良明**
(第三军医大学野战外科研究所 重庆 400042)

摘要 目的:建立一种适用于大量制备的,安全、高效的血红蛋白纯化方法。方法:将压积红细胞装入透析袋,以含有还原剂的 Tris 缓冲液透析破碎,破碎的上清经两级硫酸铵沉淀后透析至上样缓冲体系,离心后取上清即得血红蛋白提取液;红细胞提取液通过阴离子交换柱层析进一步分离,计算回收率。纯化产物浓缩后以 SDS-PAGE 及 HPLC 鉴定纯度,进行紫外-可见光谱扫描并以 ABL800 血气分析仪分析血气指标,以鲎试剂测定内毒素含量,以磷测定法测定脂质含量。结果:血红蛋白提取液中脂质去除率 98%,容易通过 0.45μm 滤膜;经阴离子交换层析纯化的血红蛋白经 SDS-PAGE(银染法)及 WB 分析没有杂蛋白条带,HPLC 分析纯度 >99%、总回收率 >85%;内毒素含量 <2 EU,高铁血红蛋白含量 <5%。结论:该血红蛋白纯化方法安全高效、成本低廉、易于放大生产,具有较好的应用前景。

关键词 血红蛋白 阴离子交换层析 硫酸铵沉淀 纯化
中图分类号 Q513; Q939.48; R394-33

由于以人红细胞为载体的传统输血方法本身存在的交叉感染、输血反应及红细胞存储期短等问题,以无基质血红蛋白为载体的新型携氧材料的开发,几十年来一直是众多医学、科研工作者研究的主要内容之一。在这个领域,发达国家处于领先的位置,有多个公司的产品进入 III 期临床。已有的以无基质血红蛋白(hemoglobin,Hb)为载体的携氧材料,包括分子内交联血红蛋白[1]、脂质体修饰的血红蛋白[2]、聚乙二醇(PEG)修饰的血红蛋白[3]、聚合物包裹的血红蛋白[4]以及基因工程重组表达的血红蛋白[5]等。除了基因工程重组表达血红蛋白以外,大部分血红蛋白都来源于动物,包括牛、猪、人等。利用这些动物源血红蛋白,首先需要将血红蛋白从红细胞中分离纯化出来。已有的血红蛋白纯化方法包括超滤法[6]、加热法[7]、萃取法[8]、柱层析法[9]。加热法、萃取法对血红蛋白的空间构象产生的影响不可避免,且萃取法引入的有机溶剂也是一种潜在的危险因素;超滤法便于进行密闭的流水线生产,为国外制药公司所广泛采用,但超滤膜需要经常更换,成本高昂,势必限制血红蛋白携氧产品的推广应用;柱层析法成本低、重复性好,对血红蛋白的空间结构没有影响,安全高效,是最有前景的血红蛋白纯化方法。

目前文献报道的血红蛋白纯化方法多采用阴离子交换柱层析法,将压积红细胞低渗破碎并高速离心取上清,再通过阴离子交换柱纯化。本实验室在采用上述方法小规模纯化血红蛋白时并未发现问题,但在放大生产时发现两个比较严重的问题。首先,红细胞膜脂质含量很高,通过离心红细胞破碎液的方式难以去除脂质。脂质与阴离子交换填料结合紧密,降低了柱效,因此通常每上样等柱床体积量的压积红细胞必须执行一次柱清洁程序。柱清洁程序耗费大量乙醇和冰醋酸,不但提高了成本,而且延长了生产周期。其次,红细胞破碎液难以直接通过 0.45μm 滤膜,频繁的滤膜更换过程增加了高铁血红蛋白(methemoglobin,metHb)的含量。本研究拟解决上述问题,建立一种安全高效的血红蛋白纯化方法。

收稿日期:2011-07-01 修回日期:2011-08-15
* 国家"973"计划资助项目(2005CB522601)
** 通讯作者,电子信箱:liangmingliu@yahoo.com

1 材料与方法

1.1 主要试剂与仪器

猪红细胞,从农户饲养家猪采集;硫酸铵,重庆博艺化学试剂有限公司;低温高速离心机,Jouan MR23i;截留分子质量5~14kDa透析袋,重庆万利实验器材有限公司;蛋白质分子质量标准,Fermentas公司;Q Sepharose Fast Flow填料,GE公司;超滤杯,截留分子质量30kDa超滤膜,上海摩速科学技术有限公司;猪血红蛋白标准品,Sigma公司;兔抗猪血红蛋白多克隆抗体SC-22718,Santa Cruz公司;300SB-C3反相柱(4.6*250mm),1200高效液相色谱仪,Agilent公司;ABL800血气分析仪,雷度公司;鲎试剂,厦门市鲎试剂厂。

1.2 实验方法

1.2.1 硫酸铵分级沉淀血红蛋白 将猪压积红细胞100ml转入透析袋,放入装有10mmol/L Tris、0.15%抗坏血酸(m/v),pH 8.0缓冲液的1L标本皿中,4℃透析3h,1h换液1次。将破碎的红细胞转入50ml离心管,边加边搅拌加入研磨的硫酸铵粉末,使其终饱和度达到0℃时饱和度为20%,4℃ 10 000g离心10min;将上层漂浮物移去,然后将中间层蛋白液转移到新的50ml离心管。向新离心管中的蛋白液加入硫酸铵粉末使其终饱和度达到0℃时饱和度46%,4℃ 4 000g离心10min;将上层液体弃去,以Binding Buffer(脱氧的25mmol/L Tris, 10mmol/L NaCl, pH8.5)洗涤沉淀2次后重悬沉淀。将硫酸铵沉淀的血红蛋白液转入透析袋,以Binding Buffer为透析液于4℃进行透析,每1h换液一次,共3次。将透析后的血红蛋白溶液按体积比1∶2以Binding Buffer稀释,4℃ 12 000g离心10min。将离心上清以0.45μm滤膜过滤后,进行阴离子交换层析。

1.2.2 阴离子交换柱层析法纯化血红蛋白 以去离子ddH_2O冲洗柱床体积为50mm×200mm($d\times h$)的Q sapharose Fast Flow层析纯化柱5倍柱床体积,以2倍柱床体积的Binding Buffer平衡柱后将以步骤1.2.1制备的血红蛋白提取液调节pH至8.5后上样,上样完成后以Binding Buffer再平衡2倍柱床体积,最后以脱氧的25mmol/L Tris、80mmol/L NaCl (pH 8.5)洗脱血红蛋白,流速皆为10ml/min。纯化产物以氩气加压的超滤杯(超滤膜截留分子质量30kDa)超滤浓缩。

1.2.3 纯化血红蛋白的鉴定 血红蛋白纯度以十二烷基磺酸钠-聚丙烯酰铵凝胶电泳(sodium dodecyl sulfate polyacrylamide gel electropheresis, SDS-PAGE)以及搭载300SB-C3反相柱的Agilent 1200高效液相色谱(high performance liquid chromatography, HPLC)进行分析;血红蛋白氧合状态通过紫外-可见光谱扫描、ABL800血气分析仪进行分析,主要考察metHb含量;通过免疫印迹法(Western blot, WB)进行定性分析;通过鲎试剂测定内毒素含量,定磷法测定脂质含量。

1.3 数据处理

数据为多次测定后采用$\bar{\chi}\pm s$表示,本方法与一般方法在除去脂质效果方面的比较采用单因素方差分析与t检验。

2 结 果

2.1 硫酸铵分级沉淀血红蛋白

以SDS-PAGE分析沉淀前后蛋白质溶液蛋白质组成的变化,结果见图1。以氰化亚铁试剂盒测定硫酸铵分级沉淀前后血红蛋白浓度,计算硫酸铵分级沉淀法的血红蛋白回收率;以中国药典纪录的定磷法测定脂质含量,各项参数见表1。结果表明,经过硫酸铵分级沉淀的血红蛋白溶液,杂蛋白得到有效的去除,从而简化阴离子交换层析步骤的操作。脂质得到有效的去除,避免了频繁的清洗阴离子交换填料的过程,节约了时间,同时降低了成本。另外,经过硫酸铵分级沉淀以后的蛋白质溶液易于通过0.45μm滤膜,有效的避免了由于频繁更换滤膜引起metHb含量升高的现象。

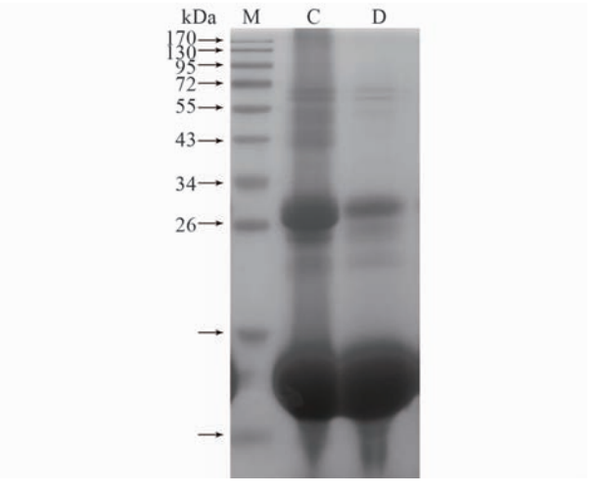

图1 SDS-PAGE分析硫酸铵分级沉淀前后蛋白质组成变化

Fig. 1 SDS-PAGE result for hemoglobin extract before and after ammonium sulfate precipitation

M: Standard molecule weight protein; C: Hemoglobin extract before precipitation; D: Hemoglobin extract after precipitation

表 1　血红蛋白硫酸铵分级沉淀各项参数表
Table 1　Parameters for ammonium sulfate precipitation of hemoglobin

项目	常规方法[a]	本方法
血红蛋白回收率	>90%	>85%
脂质含量	100%	<2%[b]
通过 0.45μm 滤膜	困难	容易

a. Conventional method: packed red blood cells were lysed by diluting with ddH_2O, and the supernatant was removed into new tubes for further purification after high - speed centrifugation; b. Lipid proteins were effectively removed in hemoglobin extracts obtained by ammonium sulfate precipitation compared with conventional method, $P<0.01$.

2.2　阴离子交换柱层析纯化血红蛋白

经过硫酸铵分级沉淀处理的血红蛋白溶液，以阴离子交换柱层析纯化，产物纯度以 SDS-PAGE（银染法）及 HPLC 鉴定。SDS-PAGE 结果显示[图 2(a)]，通过本方法纯化的血红蛋白纯度较高，优于 Sigma 标准品（纯度 >98%）；纯化血红蛋白与标准品在约 32kDa 处皆有蛋白质条带，进行 WB 分析以鉴定该蛋白质成分[图 2(b)]，结果显示分子质量约为 16kDa 和 32kDa 的蛋白质条带都是血红蛋白，目前推测分子质量约为 32kDa 的蛋白质条带由血红蛋白 α、β 亚基自身交联形成，是否如此还需要进一步验证。

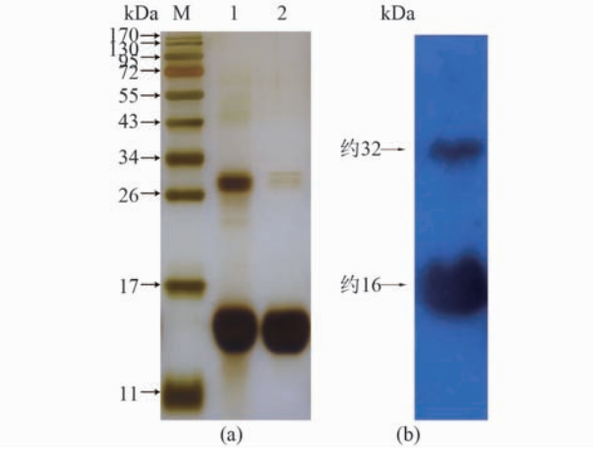

图 2　SDS-PAGE 及 WB 分析阴离子交换柱层析纯化血红蛋白的纯度
Fig. 2　Purity of the hemoglobin purified by anion exchange chromotography by SDS-PAGE and WB

(a) SDS-PAGE result by silver stain; M: Standard molecule weight protein; 1: Porcine hemoglobin standard from Sigma; 2: Hemoglobin purified by anion exchange chromatography (b) Western blot for hemoglobin purified by anion exchange chromatography, both the 16kDa and 32kDa protein bound with the anti-hemoglobin IgG specifically

HPLC 分析结果见图 3，经过硫酸铵沉淀制备的血红蛋白提取液中 Hb 的纯度已经接近 Sigma 公司的标准品[图 3(a)、(b)]，杂峰少而且拖尾峰不明显，显示不但纯度提高，而且血红蛋白一级结构未受到破坏，但血红蛋白异构体去除还不完全[图 3(c)、(d)]；经过阴离子柱层析纯化后血红蛋白质含量为 99.8%，血红蛋白异构体也基本去除[图 3(e)、(f)]，达到进行下一步实验的要求。紫外光谱扫描结果见图 4，光谱图像显示纯化产物中大部分 Hb 处于氧合状态，且在 540nm 及 590nm 处吸收峰明显，提示携氧功能正常；另外经血气分析仪测试，纯化 Hb 中 metHb 含量 <5%，显示纯化过程中 Hb 得到保护免于受到氧化破坏。

阴离子交换层析纯化的血红蛋白的各项参数见表 2，均符合中国药典关于生物制品的要求。

表 2　阴离子交换层析纯化的猪血红蛋白参数表
Table 2　Parameters of purified porcine hemoglobin by anion exchange chromatography

项目	参数
血红蛋白浓度	6(±0.1)%
血红蛋白纯度	>99%
血红蛋白回收率	90(±5)%
高铁血红蛋白含量	<5%
氧合血红蛋白含量	>95%
内毒素含量	<2EU/ml
磷脂含量	<3.3nm/ml
pH	7.3±0.1

3　讨论

以无基质血红蛋白为基础的新型携氧材料，经过几十年来众多研究者的不懈努力，已经趋于成熟。在南非等某些地区，已经有一些医院与医药公司合作开展临床应用[10]。尽管无基质血红蛋白携氧载体进入全面应用仍需进一步研究与完善，但其光明前景值得人们期待。在无基质血红蛋白携氧材料的开发与应用等各个方面，国外都处于遥遥领先的位置。为了便于进行规模化、标准化生产，国外公司多采用密闭流水线，以超滤膜结合各级过滤滤芯生产无基质血红蛋白[11]。由于红细胞膜脂质含量非常高，在进行过滤、超滤的过程中滤膜极易堵塞，需要对各级滤芯进行频繁的清洗，超滤膜也需要经常更换。因此这种生产方法成本极高，对大规模应用造成困难。对于国内，情况更是如此，因为超滤膜及各种滤芯严重依赖进口。因此，一种低成本的、便于进行标准化的无基质血红蛋白制备方法，具有重要意义。

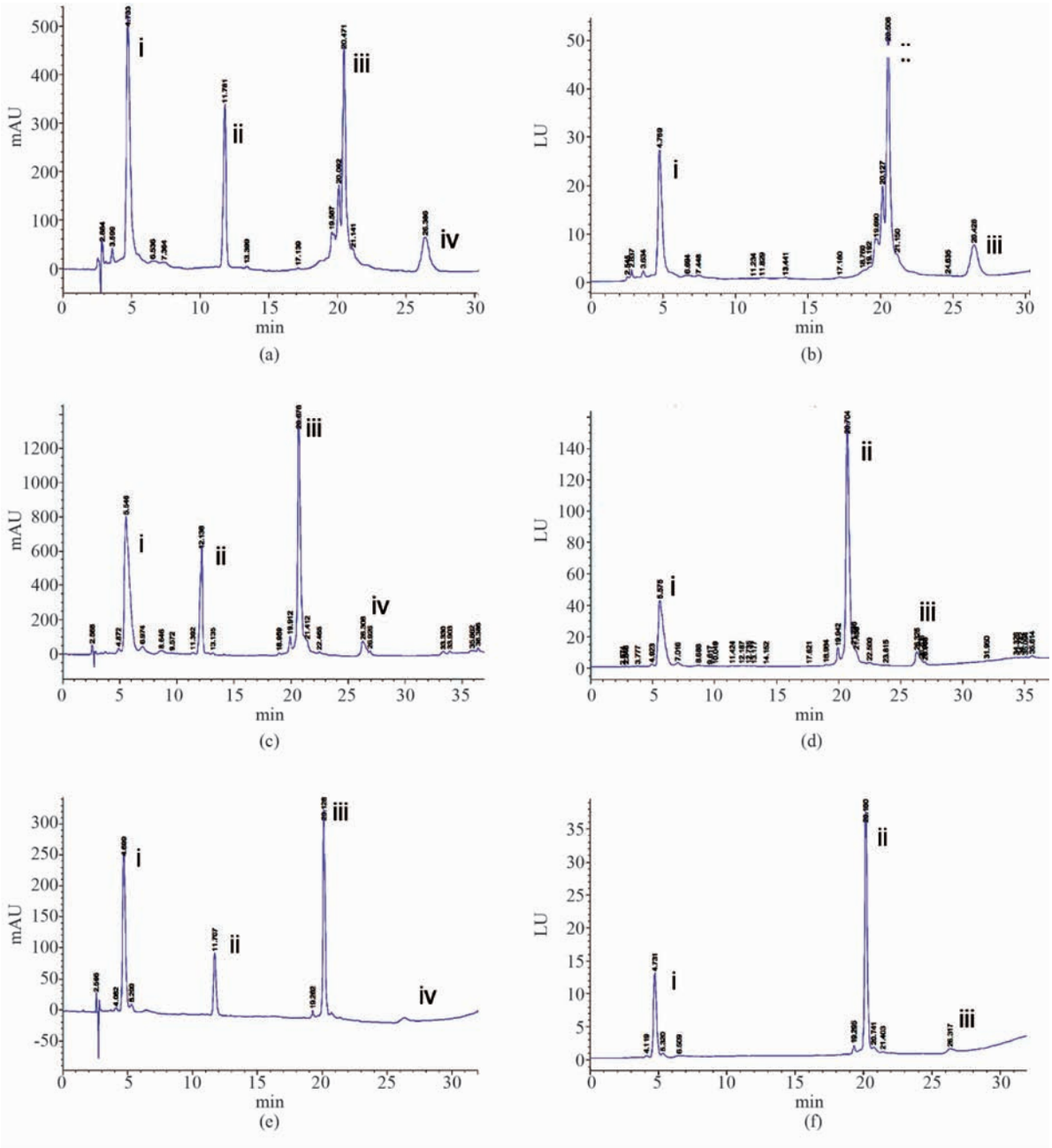

图 3 HPLC 分析纯化血红蛋白纯度

Fig. 3 Purity analysis result of purified hemoglobin by HPLC

(a),(b) Sigma porcine hemoglobin standard (nominal purity > 98%) (c),(d) unpurified porcine hemoglobin (e),(f) purified hemoglobin. (a),(c) and (e) were spectrum from VWD detector, and (b), (d) and (f) were from fluorescence detector. Samples were analyzed by the Agilent 1200 HPLC system with a Zorbax 300Extend-C18 column (4.6 * 250mm) at 25℃ and a guard column was used。Samples of 5 (l each were injected by the auto-sampler and signals were monitored by the VWD (254nm) and the FLD (EX = 250nm, EM = 340nm) detector. Acetonitrile containing 0.1% TFA (a) and ddH_2O containing 0.1% acetonitrile (b) were used as the mobile phase following the gradient as 40% 5min, 40% ~42% 5min, 42% 5min, 42% ~45% 5min, 45% 5min, 45% ~60% 5min and 60% ~80% 5min at the flow rate of 1ml/min. In the acidic mobile phase hemoglobin dissociated into [peak iii in (a),(c),(e) and peak ii in (b),(d),(f)],β(peak i in all spectrums) subunits and the heme [peak ii in (a),(c),(e)]. There was another peak [iv in (a),(c),(e) and iii in (b),(d),(f)] which was found to be a hemoglobin isoform corresponding to the 32kDa strap in SDS-PAGE

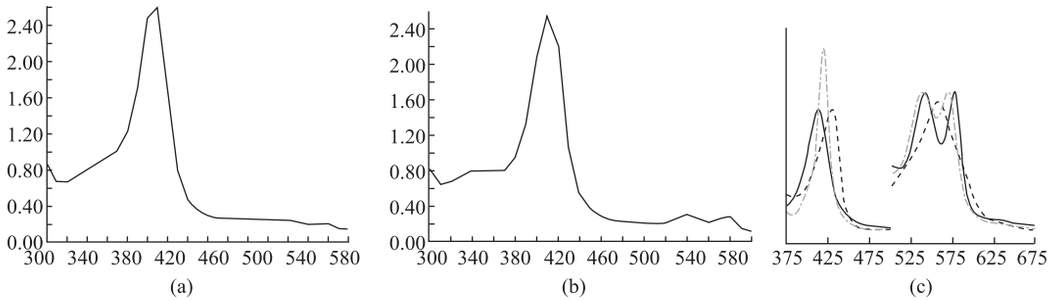

图 4　猪 Hb 标准品及纯化样品的紫外-可见光扫描光谱

Fig. 4　UV-Vis spectrums of porcine hemoglobin standard and purified hemoglobin

(a) UV-Vis spectrum of porcine hemoglobin standard (Sigma)　(b) UV-Vis spectrum of purified porcine hemoglobin. All samples were diluted by 25mmol/L Tris pH 7.4 and then scanning was performed in the range of 200 ~ 600nm with the interval of 2nm　(c) UV-Vis spectrum of hemoglobin reported by other researchers. —,---and-.-. represented oxyhemoglobin, deoxyhemoglobin and carbonmonoxy ferrous hemoglobin respectively

脂质由于其特殊的结构,通过常规的离心方式难以去除。使用有机溶剂萃取的方法可以去除脂质,但有机溶剂的引入有可能造成血红蛋白的变性,尤其是残留的有机溶剂对生物体的伤害以及对离子交换填料的损伤。如果要通过离心的方式去除脂质及磷脂,必须改变红细胞破碎液的密度,从而使血红蛋白与脂质在离心过程中处于不同的位置。硫酸铵沉淀法是常用的蛋白质粗分离方法,但在研究初期我们用这种方法去除脂质的效果并不理想,后来发现原因在于红细胞破碎的方式。目前报道的红细胞破碎采用的方法是将压积红细胞以蒸馏水稀释,造成低渗环境。但在此过程中蛋白质浓度降低,其密度不足以使脂质与血红蛋白处于离心管中的不同位置。我们研究的解决方法是将压积红细胞装入透析袋中,以含有抗坏血酸的 Tris 盐酸缓冲液透析破碎,然后加入硫酸铵进行分级沉淀。结果证明此方法不但有效地去除了脂质,控制了高铁血红蛋白的含量,而且由于红细胞破碎液体积减小,耗费的硫酸铵量控制在最小,进一步控制了成本。

本研究解决了阴离子交换柱层析法纯化血红蛋白的其中两个重要问题,没有引入有害成分,且产物纯度、血红蛋白回收率都比较高,是一种安全高效的血红蛋白纯化方法。但阴离子交换柱层析法进行血红蛋白纯化也有一些需要解决的问题,比如不易进行密闭的流水线生产,产品质量稳定性比超滤法低等,这都是进一步研究方向。

参考文献

[1] Hu D, Kluger R. Functional cross-linked hemoglobin bis-tetramers: geometry and cooperativity. Biochemistry, 2008, 47 (47): 12551-12561.

[2] Kaneda S, Ishizuka T, Goto H, et al. Liposome-Encapsulated Hemoglobin, TRM-645: Current Status of the Development and Important Issues for Clinical Application. Artif Organs, 2009, 33 (2): 146-152.

[3] Svergun D I, Ekström F, Vandegriff K D, et al. Solution Structure of Poly (ethylene) Glycol-Conjugated Hemoglobin Revealed by Small-Angle X-Ray Scattering: Implications for a New Oxygen Therapeutic. Biophysical Journal, 2008, 94(1): 173-181.

[4] Duan L, He Q, Yan X H, et al. Hemoglobin protein hollow shells fabricated through covalent layer-by-layer technique. Biochemical and Biophysical Research Communications, 2007, 354(2): 357-362.

[5] Graves P E, Henderson D P, Horstman M J, et al. Enhancing stability and expression of recombinant human hemoglobin in *E. coli*: Progress in the development of a recombinant HBOC source. Biochim Biophys Acta, 2008, 1784(10): 1471-1479.

[6] Cai J, Meng W F, Hong M, et al. Chemically modified porcine hemoglobins and their biological properties. Protein and Peptide Letters, 2004, 11(4): 353-360.

[7] Sakai H, Takeoka S, Yokohama H, et al. Purification of concentrated hemoglobin using organic solvent and heat treatment. Protein Expr Purif, 1993, 4(6): 563-569.

[8] Wiencek J M, Nabi A, Boo J Y. Hemoglobin extraction using cosurfactant-free nonionic microemulsions. Separation Science and Technology, 1994, 29(7): 923-929.

[9] Andrade C T, Barros L A M, Azero M C P. Purification and characterization of human hemoglobin: effect of the hemolysis conditions. International Journal of Biological Macromolecules, 2004, (34): 233-240.

[10] Michael L, Parchman, J A, Pugh R L, et al. Competing

demands or clinical inertia: The case of elevated glycosylated hemoglobin. Annals of Family Medicine, 2007, 5:196-201.
[11] Moore E E, Johnson J L, Moore F A, et al. The USA multicenter prehosptial hemoglobin-based oxygen carrier resuscitation trial: scientific rationale, study design, and results. Crit Care Clin, 2009,25(2):325-356.

An Efficient and Safe Method for Hemoglobin Purification

ZANG Jia-tao LIU Jian-cang XU Jing LI Dong-hong LIU Liang-ming
(Second Department of the Institute of Surgery, Third Military Medical University, Chongqing 400042, China)

Abstract Objective: To build up a hemoglobin purification method which could meet the needs of safety, efficiency and scale amplification. Methods: Packed red blood cells were lysed by dialyzing against 10 mmol/L Tris-HCl (pH 8.0) containing 0.15% (*M/V*) ascorbic acid at 4℃ for 3 h with the buffer changed once an hour, and then centrifuged at 10 000 *g* for 10 min (4℃). The supernatant was treated with ammonium sulfate by the two-step salting-out operation to the saturation of 20% and 46% (*M/V*, calibrated at 0℃) subsequently, and then the precipitated protein was re-suspended using the equalizing buffer (25 mmol/L Tris, 10 mmol/L NaCl pH 8.5) with the equal volume to that of the packed red blood cells. After dialyzing against the equalizing buffer for at 4℃ for 3 h with the same changing interval, the protein solution was diluted with the equalizing buffer by the ration of 1∶2 (*V/V*) and centrifuged at 12 000 *g* for 20 min (4℃). The supernatant after centrifugation, namely the rough hemoglobin extract, was loaded to an 50 mm * 200 mm (d * h) Q sapharose FF anion exchange chromatography column pre-equalized with the equalizing buffer and then hemoglobin was eluted using 25 mmol/L Tris, 80 mmol/L NaCl pH 8.5. The purity of samples from different processed was analyzed by SDS-PAGE and HPLC, and the oxygen-binding status was characterized by the UV-Vis spectrum scanning and blood-gas indexes, which were provided by the Thermo Multiscan Spectrum and ABL800 Flex Blood-gas Analyzer respectively. Lipoprotein concentration was tested by the phosphorus determination method and LPS was determined using the stachypleus amebocyte lysate with the sensitivity of 0.3 EU/ml, 0.1 EU/ml and 0.03 EU/ml. Results more than 98% lipids were removed in the rough hemoglobin extracts compared with hemoglobin extracts prepared by the conventional method, and this rough hemoglobin extracts could easily go through the 0.45 μm cellulose nitrate membrane. In the following anion exchange chromatography process, it could be observed that column contaminants were efficiently removed by the two-step ammonium sulfate precipitation. No other protein but hemoglobin could be detected in the hemoglobin purified by anion exchange chromatography through SDS-PAGE (silver staining) and WB, and HPLC analysis indicated a purity of above 99%. The total recovery of hemoglobin in the anion exchange chromatography was above 85%, and most hemoglobin molecules were in the ferrous oxyhemoglobin status and methemoglobin was less than 5% in the purified hemoglobin revealed by UV-Vis spectrum scanning and blood-gas determination. LPS was less than 2EU/ml. Conclusion: The hemoglobin purification method introduced above could meet the need of safety, efficiency, cost control and amplification, and a good application could be expected in the future.

Key words Hemoglobin Anion exchange chromatography Ammonium sulfate precipitation Purification