Arabian Journal of Chemistry (2014) 7, 292–296

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Determination of erythromycins in fermentation broth using liquid phase extraction with back extraction combined with high performance liquid chromatography

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Received 4 October 2010; accepted 29 October 2010
Available online 9 November 2010

KEYWORDS

Liquid phase extraction with back extraction;
Fermentation broth;
Erythromycin;
Clean-up;
High performance liquid chromatography

Abstract Liquid phase extraction with back extraction (LPE-BE) combined with high performance liquid chromatography-diode array detection (HPLC-DAD) was applied for the extraction and determination of erythromycin A, B and C in fermentation broths. According to this procedure, the fermentation broth with the adjustment pH at a fixed value of 10 was first mixed with organic solvent (V_{broth}/V_{org} = 1.0). After shaking, the mixture was separated into two phases by microfuging at 13,000 rpm for 15 min. Then back extraction was performed into the acidic aqueous phase with pH 5.0 (V_{org}/V_{aq} = 1.0). After centrifugation at 3000, the two phases were separated and 50 μL of the acidic aqueous phase was injected into the HPLC. The effects of different variables such as the nature of extraction solvent and the pH of samples and buffer were investigated. At the most appropriate conditions, dynamic linear ranges of 0.5–8, 0.1–0.9 and 0.1–0.9 mg mL^{-1} and limits of detection of 0.03, 0.003 and 0.002 mg mL^{-1} were obtained for erythromycin A, B and C, respectively. Relative standard deviations (RSDs) of the proposed method were less than 9.5%. The mean recoveries were 99.5%. The proposed method is simple and sensitive with highly clean-up effect and it can be used for monitoring the progress of erythromycin fermentation.

1. Introduction

Erythromycin is a macrolide antibiotic, produced by fermentation of Saccharopolyspora (Ribeiro and Ribeiro, 2005). Production and biosynthesis of erythromycin are extensively under investigation. (Bushell et al., 1997; Heydarian et al., 1996). The fermentation produces not only erythromycin A (EA) but also small quantities of related substances such as *N*-dimethyl EA and erythromycin B, C, D, E and F (Fig. 1).

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.arabjc.2010.10.033

Determination of erythromycin and its related substances in fermentation broth is performed by high performance liquid chromatography after a sample pretreatment step such as liquid–liquid extraction (LLE) (Tsuji and Goetz, 1978; Chepkwony et al., 2001; Trilli et al., 1987) and solid phase extraction (SPE) (Heydarian et al., 1998). By using LLE as a pretreatment procedure, other materials in fermentation samples such as proteins, nutrient, oil and impurities, along with erythromycin, might be extracted. Therefore, LLE is not a sensitive method for complex matrixes. SPE is a time-consuming method in which a solvent evaporation step is required in order to preconcentrate the analytes before final analyses (Ebrahimzadeh et al., 2007). Liquid phase extraction with back extraction (LPE-BE), a three phase extraction technique, is suitable for purification of ionizable compounds. It also overcomes many drawbacks of conventional LLE or SPE methods as it is simple, inexpensive, fast and sensitive. In this study, the sample is adjusted to pH, where the analytes are in their neutral forms. Then by mixing sample with organic solvent, the neutral molecules are transferred into the organic phase. During back extraction, the neutral molecules, present in the organic phase, are ionized with the acceptor solution. By shaking, ionizable compounds are extracted into the organic solvent and then back extracted into the acceptor solution, which can be directly analysed. The simplicity of operation, short time, high sensitivity and recovery are the advantages of LPE-BE.

The aim of the present study was to investigate the feasibility of LPE-BE to extract and determine erythromycin A and its related substances in fermentation broth. Various parameters including type of organic solvent, pH of sample and buffer solution on the extraction efficiency were investigated and optimized.

2. Experimental

2.1. Reagents

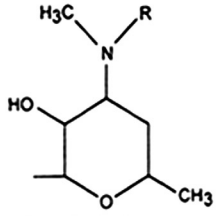
Anuh pharma LTD (Mumbai, India) reference standard of erythromycin base containing 93.5% erythromycin A and USP reference standard (RS) of erythromycin B and C were used throughout the study. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Fluka (Buchs, Switzerland). HPLC grade acetonitrile was purchased from Caledon (Georgetown, Ont., Canada). Ammonia solution 25%, dibuthyl ether, *n*-butylacetate, and ethyl acetate were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared using a Milli-Q system from Millipore (Bedford, MA, USA). Fermentation broth samples were obtained from Shafa-e-Sari Company (Tehran, Iran).

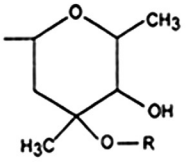
2.2. Preparation of standard solutions and real samples

Stock standard solutions containing 10 mg mL^{-1} of erythromycin A, 1 mg mL^{-1} of erythromycin B, 1 mg mL^{-1} of eryth-

Analyte	R1	R2	R3	R4
Erythromycin A	DS	CD	OH	H
Erythromycin B	DS	CD	H	H
Erythromycin C	DS	MR	OH	H

R





CH_3 Desosamine (DS) Cladinose (CD)

H Mycarose (MR)

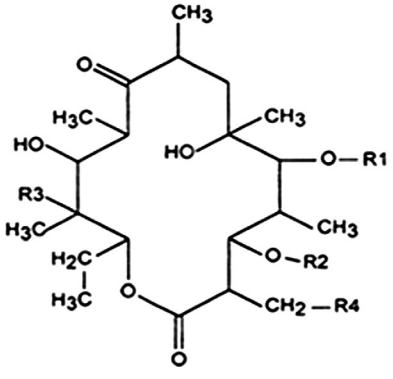


Figure 1 Chemical structures of EA, EB and EC.

romycin C were prepared in acetonitrile and stored in a fridge at 4 °C. Stability of standard solution (3 days) was tested at +4 °C temperature and the result showed that concentration changes during storage were less than 0.5%. The working solutions were prepared daily by diluting the standard solutions prior to use. Free erythromycin fermentation broth samples and the industrial fermentation broth containing about 5–8 mg mL^{-1} erythromycin were used.

2.3. Apparatus

Injection of the samples into high performance liquid chromatography (HPLC) was carried out using a 50-μL Hamilton (Bonaduz, Switzerland). Separation and quantification of erythromycin and its related substances were carried out using Shimadzu LC-10AD VP HPLC system from Shimadzu Company (Kyoto, Japan) equipped with a diode array detector. LC-solution was used for the acquisition and processing of the data. Chromatographic separations were carried out using a Shim-Pack CLC-C_{18} analytical column (250 × 4.6 mm I.D., 5 μm) from Shimadzu (Kyoto, Japan). A mixture of 10 mmol L^{-1} of potassium dihydrogen phosphate buffer and acetonitrile (55:45) adjusted to pH 7.0 and a flow rate of 1.0 mL min^{-1} was used as a mobile phase in isocratic elution mode. The injection volume was 50 μL for all the samples and the detection was performed at the wavelength of 205 nm. The column temperature was controlled at 50 °C by oven column. Three blank samples of standard solution were analysed after injection of high concentration of erythromycins. The results showed no memory effect on HPLC column. Eppendorf microfuge model 5415C (Golden valley, MN, USA) and Hettich centrifuge model EBA 20 (Oxford, England) were employed for phase separation.

2.4. LPE-BE procedure

500 μL of fermentation broth sample with pH 10 containing erythromycin was placed in a 1.5 mL micro tube. 500 μL of *n*-butylacetate (as extraction solvent) was added into the sample. The mixture was gently shaken for 5 min. The mixture was then microfuged at 13000 rpm for 15 min. Then the organic phase containing the analytes was mixed with 500 μL acceptor solution with pH 5. After centrifuging at 3000 rpm for 5 min, the two phases were separated and 50 μL of the acceptor solution was injected into HPLC. The entire scheme of the procedure is shown in Fig. 2.

3. Results and discussion

3.1. Study of the experimental parameters

The important aims of the three phase LPE are to separate and clean-up the analytes from the complex matrixes and to provide efficient pretreatment prior to HPLC determination. It involves the extraction of ionizable compounds from the aqueous sample at suitable pH into the organic solvent, followed by back-extraction into the receiving aqueous phase by adjusting the pH to the desired value. In this study, LPE-BE combined with HPLC-DAD was developed for extraction and determination of erythromycin A, B and C in fermentation broths. In order to achieve maximum extraction efficiency, various parameters affecting the extraction efficiency were optimized. The optimization was carried out by free erythromycin fermentation broth samples with addition of 5,

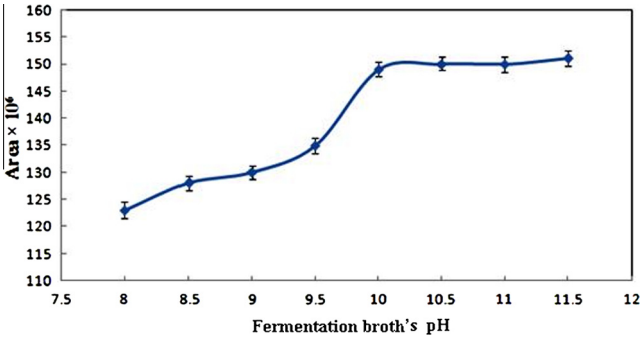


Figure 3 Effect of fermentation broth's pH on the extraction of analytes. 500 μL broth containing 5, 0.5 and 0.5 mg mL^{-1} of EA, EB and EC, respectively, was extracted using *n*-butylacetate, then back extracted into a buffer KH_2PO_4 (25 mM) adjusted to pH 6.

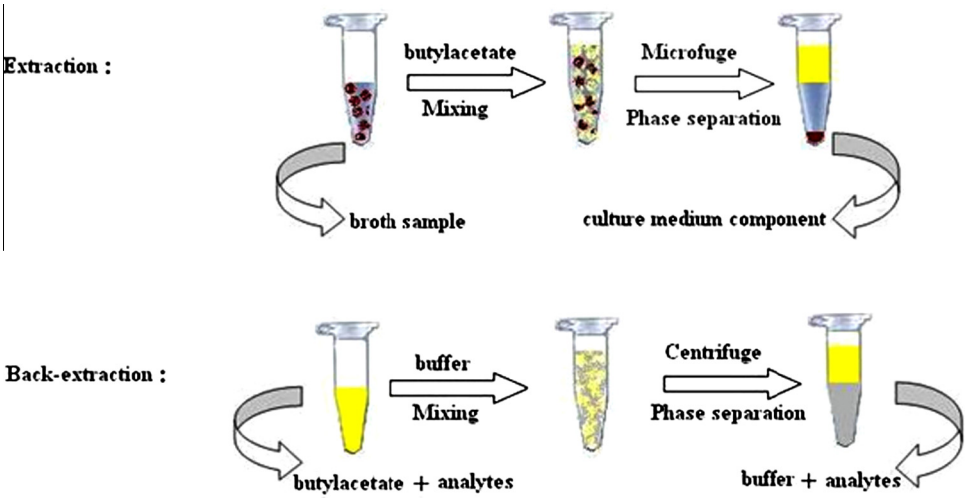


Figure 2 Scheme of LPE-BE technique for erythromycins.

0.5, and 0.5 mg mL^{-1} of erythromycin A, B and C, respectively.

3.1.1. Selection of extraction solvent

Extraction efficiency is directly influenced by the nature of the organic solvent. Thus, the organic solvent requires characteristics such as immiscibility with sample solution, low volatility and higher affinity to the drug (He and Kang, 2006). In fermentation broth, there exist some components in culture medium such as proteins, polysaccharides, and nutrient which would sediment during microfugation. Therefore, it is better to use a solvent with lower density than water. In this case after microfugation the organic solvent floats on the sample. Considering these facts, dibutyl ether, *n*-butylacetate, and ethyl acetate were tested for their suitability. Among these solvents, *n*-butylacetate offered the highest efficiency. Therefore, it was used for subsequent studies.

3.1.2. Effect of fermentation broth's pH

Fermentation broth's pH has a very important role in the extraction efficiency. Since, EA is a weak basic compound (pK_a 8.25) (Galichet, 1986), the drug was deionized under basic conditions in sample and its solubility in the sample was reduced. Thus, by mixing sample with organic solvent, erythromycin and its related substances were extracted into the organic phase. The initial pH of the fermentation broth is in the range of 4.6–6.6. Therefore, extraction of the analytes was performed in the range of pH = 8–12, using concentrated ammonia solution (25% w/w). As shown in Fig. 3, the peak area rises by increasing the pH value. Therefore, the pH of the fermentation broth was adjusted to 10 in further studies.

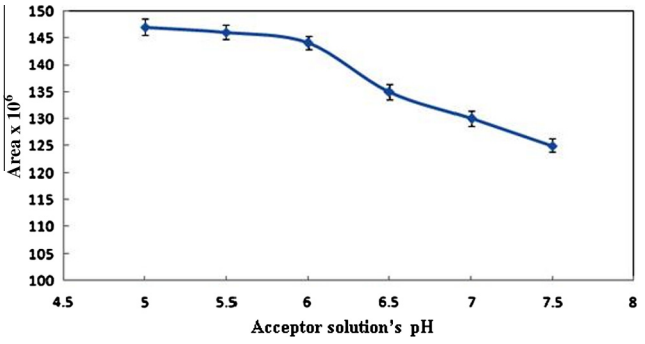


Figure 4 Effect of acceptor solution's pH on the extraction of analytes. 500 μL broth containing 5, 0.5 and 0.5 mg mL^{-1} of EA, EB and EC, respectively, at pH 10 was extracted using *n*-butylacetate, then back extracted into the acceptor solution.

3.1.3. Effect of acceptor solution's pH

For the back-extraction process, the neutral molecules, present in the organic phase, should be extracted into the acceptor solution by protonation of the analytes. In this case, the acceptor solution's pH should be acidic in order to ionize the analytes. Therefore, the effect of acceptor solution's pH on the transport efficiency was investigated in the range of 5–7.5 using KH_2PO_4 (25 mM) buffer. Since, erythromycin is prone to degradation at lower pH, therefore, pH < 5.0 was not tested. As shown in Fig. 4 the maximum peak area was obtained at pH 5.0. at higher values, the extraction efficiency decreased due to the incomplete protonation of the analytes. Thus, the pH of buffer solution was adjusted to 5.0 in further studies.

3.1.4. Effect of ionic strength

The effect of ionic strength on extraction efficiency was examined by adding sodium chloride to fermentation broth samples at the percentage levels of 0–10% (W/V). The results showed that no considerable change in the extraction efficiency was observed. So, all the subsequent experiments were performed in the absence of salt.

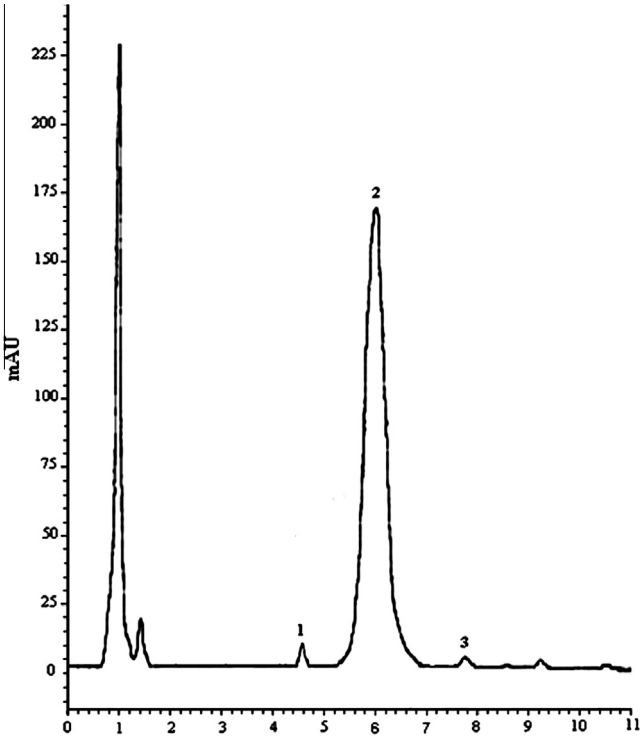


Figure 5 Chromatogram of fermentation broth sample after LPE-BE under optimum conditions. Peak: (1) EC; (2) EA; (3) EB.

Table 1 Figures of merit of the proposed method.

Analyte	LOD (mg mL^{-1})	r^2	Regression equation	DLR (mg mL^{-1})	Extraction recovery (%)
Erythromycin A	0.03	0.9994	$Y = 1636.1\ C^{a} + 1155$	0.5–8	99.5
Erythromycin B	0.003	0.9986	$Y = 2460.3\ C + 5921$	0.1–0.9	99.2
Erythromycin C	0.002	0.9994	$Y = 1807\ C + 197.61$	0.1–0.9	99.4

[a] Concentration in mg mL^{-1}.

Table 2 Determination of erythromycins in fermentation broth of Shafa-e-Sari Pharmaceutical Company (Iran).

Sample	Analyte											
	Erythromycin A				Erythromycin B				Erythromycin C			
	C_{added} (mg mL^{-1})	C_{found} (mg mL^{-1})	RSD (%)	E (%)	C_{added} (mg mL^{-1})	C_{found} (mg mL^{-1})	RSD (%)	E (%)	C_{added} (mg mL^{-1})	C_{found} (mg mL^{-1})	RSD (%)	E (%)
1	–	4.32	7.5	–	–	0.72	8.1	–	–	0.29	8.9	–
	4.0	7.92	7.6	−10.0	1.0	1.61	8.4	−11.0	0.5	0.74	9.1	−10.0
2	–	4.80	7.8	–	–	0.78	8.9	–	–	0.33	8.8	–
	4.0	8.35	8.1	−11.2	1.0	1.68	8.1	−10.0	0.5	0.88	8.7	10.0
3	–	4.60	8.2	–	–	0.75	7.9	–	–	0.34	9.5	–
	4.0	8.15	7.9	−11.3	1.0	1.84	8.4	9.0	0.5	0.89	9.3	10.0

3.2. Evaluation of the method performance

Under optimum conditions, limits of detections (LODs), regression equations, correlation coefficients (r^2), dynamic linear ranges (DLRs) and extraction recoveries (ERs) were obtained (Table 1). The LODs were calculated as the analytes concentration equal to three times of the standard deviation of the blank signal divided by the slope of the calibration curve. The extraction recovery (R) was expressed as the percentage of total analyte amount, which was transferred to the acceptor phase at the end of the extraction.

The analytes showed good linearity with correlation of determination in the range of 0.9986–0.9994. The applicability of the extraction method to real samples was examined by extraction and determination of EA, EB and EC in the industrial fermentation broths of Shafa-e-Sari Company (Tehran, Iran).

A chromatogram of the fermentation broth after LPE-BE at the optimum condition is shown in Fig. 5. Table 2 shows that the results of four replicate analytes of each sample obtained by the proposed method and the amount of added analytes are in satisfactory agreement with each other. Percent errors for determination of the analytes in different fermentation broth samples were located in the range of 9.0–11.3. On the other hand, the proposed method revealed suitable reproducibility with RSD values in the range of 7.5–9.5%.

4. Conclusions

The proposed liquid phase extraction with back extraction is a very fast and simple method for extraction and determination of milligram per milliliter concentration of erythromycin from fermentation broth. There is no need of dedicated and expensive apparatus. Simplicity and cost-effectiveness of the proposed method make it quite attractive when compared to LLE or SPE. The most important advantage of the proposed method is the ability of separation and purification of the analytes from complex matrixes. This technique is rapid, economical, applicable and suitable for the routine quality control analysis of erythromycin in fermentation broth.

Acknowledgement

This study was supported by the Vice Research of Shafa-e-Sari Company.

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