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Determination of ternary mixtures of penicillin G, benzathine and procaine by liquid chromatography and factorial design study

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Abstract

In the present work, a rapid and sensitive method for simultaneous determination of penicillin G (PG), benzathine (BE) and procaine (PR) in drug and serum media is introduced. The polar hydro-organic (55/45) mobile phases containing an aqueous solution adjusted to pH = 3.7 and an organic solvent (MeOH) including triethylamine (TEA) and trifluroacetic acid (TFA) are used. The flow rate of 1 ml min^{-1}, a C_8 column (150 mm × 46 mm) with 5 μm i.d. and wavelength at 215 nm are selected for optimal separation condition. The limit of detection (LOD), linear concentration range and relative standard deviation (R.S.D.) of this method for the PG are 1.1 μg ml^{-1}, 10–2400 μg ml^{-1} and 1.7% and for the BE are 1.2 μg ml^{-1}, 12–2100 μg ml^{-1} and 1.8% and for the PR are 1.5 μg ml^{-1}, 20–2000 μg ml^{-1} and 2%, respectively. The factorial design is used for the determination of main and interaction effects of pH, flow rate and concentration of MeOH, TEA and TFA in the separation at two levels. Also, the analysis of variance (ANOVA) table is obtained. The results show that TFA and TEA have higher effect than concentration of MeOH, pH and flow rate factors.

Keywords: Penicillin G; Benzathine; Procaine; HPLC; Factorial design

1. Introduction

Penicillins a non-toxic class of antibiotics, which are used for most infections, caused by Gram-negative cocci and majority Gram-positive bacteria [1]. Sodium salt of penicillin G (PG) reaches a maximum of plasmatic concentration in 15 or 30 min. Therefore, the use of the long-lasting penicillins like benzathine (BE) penicillin and procaine (PR) penicillin are necessary. Procaine penicillin G forms a tissue deposit, from which the drug is slowly absorbed within 12 or 24 h. Also, benzathine penicillin G is a long-acting agent [2,3]. Iranian commercialized injection of BE, PR and PG is named Pen 6:3:3.

Several methods for determination of PG, PR and BE, such as spectroscopic and electrochemical methods, have been introduced [4–8]. Although the direct electrochemical methods have rapid responses and can be used for automation, they show low selectivity. The spectroscopic methods have low selectivity for these compounds in the mixed forms. Thus, the derivative spectroscopy methods with lower sensitivity should be used [4,5]. Liquid chromatography (LC) has been widely used to improve efficiency, provide unique selectivity and extend the range of separation of these compounds in various media [9–12]. The common chromatographic methods, which were introduced for the

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simultaneous determination of PG, BE and PR needed a high column temperature (40–50 °C). Also, the shape of the peaks in these methods, especially in serum sample, is not suitable (asymmetric).

Recently, a polar organic mobile phase which combined the organic solvent with small amounts of acetic or trifluoroacetic acid (TFA), in combination with triethylamine (TEA) or ammonium hydroxide, was introduced [13–18]. This mobile phase can limit the interaction between polar compounds and the silanol-free group of stationary phase allowing a better resolution and symmetric peaks.

The determination of PG and its degradation products, 6-aminopenicilloic acid and phenyl acetic acid have been reported by LC and pyrolysis-mass spectrometry was also reported [19]. In the present work, the determination of PG, PR and BE using a polar hydro-organic solvent and C_8 column is introduced and the results are compared with the USP method [20]. The effects of pH, concentration of TEA and TFA, methanol contents and flow rate of mobile phase have been studied by factorial design. Also, ANOVA table of these factors is introduced and discussed.

2. Experimental

2.1. Instrumentation

A Shimadzu LC system (Kyoto, Japan) consisting of a SCL-6A system controller, two LC-6A pumps, a CTO-10SVP column oven, an UV–vis detector model 1602P and a C_8 column, 150 mm × 4.6 mm Lichrospher with particle diameter 5 μm were used. A Millipore Elix 5 (Millipore, USA) deionized water was used.

Table 1
The factor levels influence on separation

Factor	Unit	–	+
pH	-	3	4.4
TEA	mM	0	8
TFA	mM	0	60
MeOH	%	45	70
Flow rate	ml min^{-1}	0.8	1

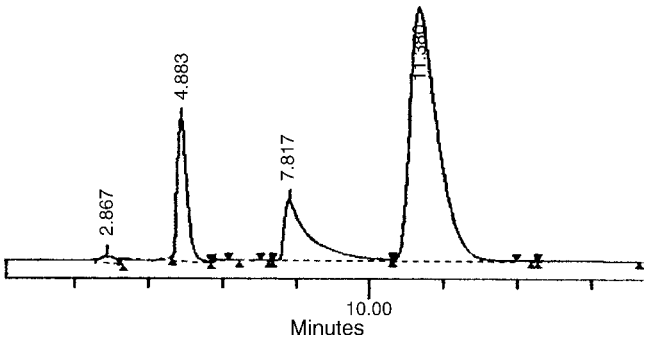

Fig. 1. The chromatogram obtained with the USP method, μ-bondapak (C_{18}) column, 50 °C. Flow rate 1.1 ml min^{-1}, wavelength 215 nm and MeOH/phosphate buffer at pH = 3.7 (30/70). The peaks at 4.883, 7.817 and 11.361 belong to PR, BE and PG, respectively.

2.2. Reagents

Iranian commercialized injection is named Pen 6:3:3 in which mix of benzathine penicillin G (600,000 units/vial), potassium penicillin G (300,000 units/vial) and procaine penicillin G (300,000 units/vial, each unit equal 1 μg). PG, BE (penicillin G bnzathine salt hydrochloride) and PR ((procaine hydrochloride 99%) were purchased from Aldrich (Taufkirchen, Germany). Triethylamine, trifluroacetic acid and sodium dihydrogen phosphate were obtained from Merck

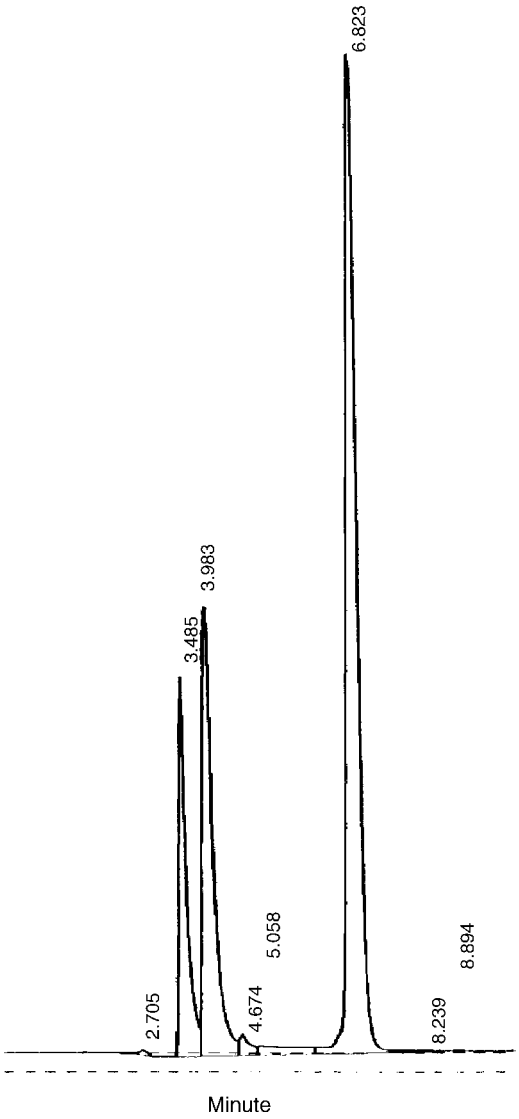

Fig. 2. The influence of 60 mM of TFA in separation of PG, BE and PR. The chromatogram is obtained on a C_8 column, at ambient temperature, flow rate 1 ml min^{-1}, wavelength 215 nm and MeOH/phosphate buffer at pH = 3 (70/30). The peaks at 3.485, 3.983 and 6.823 belong to PR, BE and PG, respectively.

(Darmstadt, Germany). HPLC-grade methanol was obtained from Fluka (Taufkirchen, Germany).

2.3. Procedure

2.3.1. Standards

Stock PG, PR and BE were prepared according to USP instruction by diluting in 40% MeOH/60% phosphate buffer pH = 7 to a concentration of 30 mg ml^{-1}. Working standards were made by dilution of a stock solution to a range of 0.5–3000 μg ml^{-1}. Standards and working solutions were aliquots in 2 ml vials and frozen until use.

2.3.2. Sample preparation

Phosphate buffer was prepared according to USP instruction by dissolving 1.4 g KH_2PO_4 and 1.3 g Na_2HPO_4 in demi-water dilution to 1 l. Mobile phases were prepared in deionized water and filtered through 0.45 μm filters before use and degassed by sonication. μ-Bondapak (C_{18}, 25 cm × 4.6 cm) column was used for USP method. Also, other solutions of mixture of TEA and TFA in MeOH were prepared. The pH of buffer solution was adjusted by 1 M H_3PO_4 or NaOH and introduced in Table 1. The mobile phase was prepared by mixing of selected ratio of buffer and MeOH. The volume of loop and analysis temperature was 20 μl and ambient temperature, respectively.

Blood was immediately centrifuged and the serum supernatant was added to equivolume of MeOH (5 ml of each) in 12 ml glass tubes supplied with Teflon-covered screw-cup. The samples were deproteinized by vortex-mixing on a multitube vortexer for 30 s. After centrifugation at 3500×g for 4 min at room temperature, the upper layer was transferred into a clean 10 ml glass tube and used to prepare calibration samples.

3. Results and discussion

The chromatogram based on the USP method for the determination of PG, BE and PR is shown in Fig. 1. The peaks at 4.833, 7.017 and 11.380 min belong to PR, BE and PG, respectively. The usual method needs a high temperature (50 °C) for the C_{18} column and the peak shapes for BE and PG are not suitable (tailing factor > 1.5). Therefore, the quantitative works for real samples have high standard deviation (relative standard deviation, R.S.D. > 1 for each of them).

Table 2
The effect of selected factor on resolutions

Run No	P	R	M	E	F	$R^{1,2}$	$R^{2,3}$	R^*
1	–	–	–	–	–	1.2	2.9	3.5
2	+	–	–	–	–	2.4	1.8	4.1
3	–	+	–	–	–	1.8	1.6	2.9
4	+	+	–	–	–	2.0	1.4	2.8
5	–	–	+	–	–	0.6	0.9	0.6
6	+	–	+	–	–	0.9	1.2	1.1
7	–	+	+	–	–	0.5	0.8	0.4
8	+	+	+	–	–	1.1	1.3	1.1
9	–	–	–	+	–	0.4	0.7	0.3
10	+	–	–	+	–	0.3	0.8	0.2
11	–	+	–	+	–	0.5	0.7	0.4
12	+	+	–	+	–	0.2	1.0	0.2
13	–	–	+	+	–	3.2	3.9	9.3
14	+	–	+	+	–	3.8	2.2	8.4
15	–	+	+	+	–	3.4	1.9	6.5
16	+	+	+	+	–	3.3	3.0	10.0
17	–	–	–	–	+	3.8	2.8	10.6
18	+	–	–	–	+	4.2	3.1	13.0
19	–	+	–	–	+	3.4	2.6	8.8
20	+	+	–	–	+	4.0	2.8	10.9
21	–	–	+	–	+	3.0	2.0	5.8
22	+	–	+	–	+	3.2	2.1	6.7
23	–	+	+	–	+	2.9	1.9	5.5
24	+	+	+	–	+	3.0	2.0	6.1
25	–	–	–	+	+	3.1	15.4	47.9
26	+	–	–	+	+	3.3	16.8	55.4
27	–	+	–	+	+	3.1	13.3	13.3
28	+	+	–	+	+	3.1	15.4	47.9
29	–	–	+	+	+	3.1	5.2	16.1
30	+	–	+	+	+	3.2	5.8	18.7
31	–	+	+	+	+	3.0	5.3	16.2
32	+	+	+	+	+	3.1	5.5	16.8

$R^{n,n+1}$, the resolution between the two near peaks, such as R^{12} and R^{23}, in which resolution between PR:BE and BE:PG, respectively, in Fig .1 and w is the peak width at the baseline.

In the present work, we used a C_8 column as stationary phase and a polar hydro-organic mobile phase containing MeOH and aqueous solution of TEA and TFA for separation of PG, BE and PR.

Full factorial design in two levels (Table 1) was used for determination of main and interaction effects of flow rate, pH, concentrations of MeOH, TFA and TEA on resolution of this mixture.

3.1. Factorial design

The influences of significant factors (pH, flow rate, concentration of TEA, TFA and methanol content in the mobile phase) have been studied. At first, TEA is added to the mobile phase and the results were investigated, then TFA is added to this mobile phase, therefore pH is changed and the influence of pH shows the effect of TFA. The experimental design approach was employed and a 2^K factorial design was run, where 2 stands for a variable level and K is the number of factors studied. The high and low values were determined and assigned as + and – coded levels, respectively, and are shown in Table 1.

The influence of these factors on the separation of PG, BE and PR can be monitored by investigation of their resolution.

$$R^{n,n+1} = \frac{t^{n+1} - t^n}{(w^{n+1} + w^n)}$$

$R^{n,n+1}$ shows the resolution between the two near peaks, such as R^{12} and R^{23} in which resolution between PR:BE and BE:PG, respectively in Fig. 1 and w is the peak width at the baseline. We used a multiplication of two obtained resolutions (R^*) as the other suitable response.

$$R^* = (R^{12})(R^{23})$$

Table 2 (Yates' method) shows the influence of variation of factors on R^*. The first divisor is the number of experiments (32 in this case).

All the other divisors are half this number. Therefore, a further column giving all the estimated effects is produced by dividing the number in the last column by its divisor. Yates' method can also provide the sum of the square values [21]. These are obtained by squaring the values in the final column and dividing by the number of experiments. The main and interactive effects of flow rate, TEA and TFA concentrations, pH and MeOH% are shown in Table 3 ($P = 0.05$).

ANOVA table allows us to verify whether the predictor variables can explain a significant amount of the variance in the response variable. Table 4 shows ANOVA table of effects

Table 3
The results obtained based on Yates' method

Run	P	R	M	E	F	Y	1	2	3	4	Divisor	Estimate	Sum of square
1	–	–	–	–	–	3.48	7.54	13.22	16.30	51.59	32	11.869	–
2	+	–	–	–	–	4.06	5.68	3.08	35.29	328.22	16	1.684	22.69695
3	–	+	–	–	–	2.88	1.60	1.09	67.48	4.03	16	−1.471	17.30190
4	+	+	–	–	–	2.80	1.48	34.20	260.74	22.92	16	−0.012	0.00113
5	–	–	+	–	–	0.55	0.54	43.34	1.68	−3.23	16	−7.579	459.57540
6	+	–	+	–	–	1.05	0.55	24.14	2.35	−20.30	16	−0.643	3.30888
7	–	+	+	–	–	0.40	17.73	192.89	5.98	3.73	16	0.959	7.36320
8	+	+	+	–	–	1.08	16.47	67.85	16.94	−3.92	16	0.298	0.71103
9	–	–	–	+	–	0.29	23.61	0.50	−1.98	22.97	16	13.266	1407.81445
10	+	–	–	+	–	0.25	19.73	1.18	−1.25	−144.24	16	0.727	4.22678
11	–	+	–	+	–	0.35	12.55	−0.19	−4.84	3.41	16	−0.618	3.05663
12	+	+	–	+	–	0.20	11.59	2.54	−15.46	−13.70	16	0.131	0.13650
13	–	–	+	+	–	9.31	103.30	4.48	−0.48	0.47	16	−3.912	122.42213
14	+	–	+	+	–	8.42	89.59	1.50	4.21	14.88	16	−0.356	1.01175
15	–	+	+	+	–	6.52	34.80	13.83	−0.66	5.27	16	0.377	1.13628
16	+	+	+	+	–	9.95	33.05	3.11	−3.26	−0.50	16	0.181	0.26100
17	–	–	–	–	+	10.59	0.58	−1.86	−10.14	18.99	16	17.289	2391.37990
18	+	–	–	–	+	13.02	−0.08	−0.12	33.11	103.26	16	1.181	11.15100
19	–	+	–	–	+	8.84	0.50	0.01	−19.20	0.67	16	−1.067	9.10578
20	+	+	–	–	+	10.89	0.68	−1.26	−125.04	10.96	16	−0.478	1.82883
21	–	–	+	–	+	5.83	−0.04	−3.88	0.68	0.73	16	−10.451	873.72450
22	+	–	+	–	+	6.72	−0.15	−0.96	2.73	−10.62	16	−1.069	9.14850
23	–	+	+	–	+	5.49	−0.89	−13.71	−2.98	4.69	16	0.901	6.48900
24	+	+	+	–	+	6.10	3.43	−1.75	−10.72	−2.60	16	−0.361	1.04040
25	–	–	–	+	+	47.86	2.43	−0.66	1.74	43.25	16	10.892	949.06353
26	+	–	–	+	+	55.44	2.05	0.18	−1.27	−105.84	16	0.643	3.30888
27	–	+	–	+	+	41.67	0.89	−0.11	2.92	2.05	16	−0.709	4.02570
28	+	+	–	+	+	47.92	0.61	4.32	11.96	−7.74	16	−0.456	1.66075
29	–	–	+	+	+	16.41	7.58	−0.38	0.84	−3.01	16	−9.318	694.61963
30	+	–	+	+	+	18.66	6.25	−0.28	4.43	9.04	16	−0.612	2.99513
31	–	+	+	+	+	16.23	2.52	−1.33	0.10	3.59	16	0.753	4.53758
32	+	+	+	+	+	16.82	0.59	−1.93	−0.60	−0.70	16	−0.268	0.57513

Y: R_a, P: pH, M: MeOH, R: Flow rate, E: TEA, F: TFA.

Table 4
ANOVA Table of data of Table 3

Factor	d.f.	SS	MS	Variance ratio
P	1	22.69695	22.69695	0.217
R	1	17.30190	17.30190	0.166
PR	1	0.00113	104.49653	–
M	1	459.57540	459.57540	4.398
PM	1	3.30888	104.49653	–
RM	1	7.36320	104.49653	–
PRM	1	0.71103	104.49653	–
E	1	1407.81445	1407.81445	13.472
PE	1	4.22678	104.49653	–
RE	1	3.05663	104.49653	–
PRE	1	0.13650	104.49653	–
ME	1	122.42213	104.49653	–
PME	1	1.01175	104.49653	–
RME	1	1.13628	104.49653	–
PRME	1	0.26100	104.49653	
F	1	2391.37990	2391.37990	22.885
PF	1	11.15100	104.49653	–
RF	1	9.10578	104.49653	–
PRF	1	1.82883	104.49653	–
MF	1	873.72450	104.49653	–
PMF	1	9.14850	104.49653	–
RMF	1	6.48900	104.49653	–
PRMF	1	1.04040	104.49653	–
EF	1	949.06353	104.49653	–
PEF	1	3.30888	104.49653	–
REF	1	4.02570	104.49653	–
PREF	1	1.66075	104.49653	–
MEF	1	694.61963	104.49653	–
PMEF	1	2.99513	104.49653	–
RMEF	1	4.53758	104.49653	–
PRMEF	1	0.57513	104.49653	–

P: pH, M: MeOH, R: Flow rate, E: TEA, F: TFA, d.f.: degree of freedom, SS: sum of squares, *MS*: mean square.

of pH, flow rate and concentration of MeOH, TEA and TFA in the separation at two levels.

3.2. Additives

3.2.1. The influence of TFA

The interaction of acids with the silica surface is greatly reduced by deliberately adding a small amount of a carboxylic acid to the mobile phase. Fig. 2 shows that the presence of TFA, changes the sequence of this mixture from PR, BE and PG to USP method. Table 3 shows that TFA has the highest effect on R^* (variance ratio = 22.885). In the presence of TFA, PPG peak appears after 20 min. The PG degrades in this condition after 6 h.

This phenomenon can be related to the acidic condition of the mobile phase, which causes an increase of interaction between silanol group and acidic compounds.

3.2.2. The influence of TEA

The addition of appropriate mobile phase modifiers can eliminate problems due to secondary interactions of basic compounds with acidic silanols. These modifiers preferentially block the silanols that cause secondary retention. The presence of TEA in the mobile phase, causes the decrease of the interaction of PPG and free silanol groups in stationary phase. Fig. 3 shows these phenomena.

Fig. 3 shows that the presence of TEA in mobile phase inversed the sequence peaks of USP method (Fig. 1). Table 4 shows this presence as secondary effective parameter (variance ratio = 13.472).

3.3. Influence of the composition of the mobile phase

All the separations were carried out with RP mobile phase containing water–methanol and organic modifiers. It appears that their retention times decreases when methanol content in mobile phase is increased. But the resolution of these compounds decreases with increase of methanol content. ANOVA table shows that the content of MeOH in mobile phase is a significant effective parameter (variance ratio = 4.398).

3.4. Influence of pH

Table 3 shows that the pH not significantly affects resolution. Correct buffer preparation is a key factor in the separation. In this work, the aqueous phase is adjusted at suitable pH and then organic solvent, containing of TEA and TFA, is added to the aqueous phase.

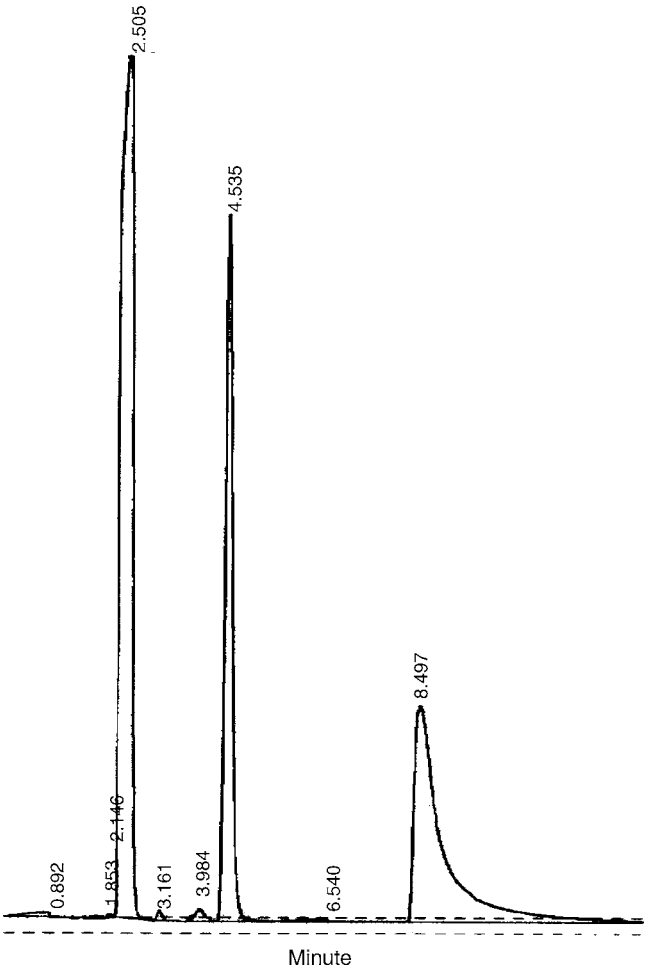

Fig. 3. Chromatogram of PR, BE and PG in phosphate buffer/MeOH (30/70), 8 mM TEA, ambient temperature, wavelength 215 nm, flow rate 1 ml min^{-1} and C_8 column. The peaks at 2.505, 4.535 and 8.497 belong to PG, BE and PR, respectively.

3.5. Flow rate

The optimum condition of flow rate is related to the number of theoretical plate and resolution is required for separation. We investigated two levels 0.8 and 1 ml min^{-1} of flow rate (Table 1). The influence of flow rate on the separation condition is shown in Table 3. Table 4 shows that the flow rate is not significant effects on R^* in this conditions.

Fig. 4 shows the influence of a mixture of TFA and TEA in an aqueous/MeOH (45:55). The optimum condition of separation can be obtained by simplex method that requires long time for analysis. Thus, we obtained nearly optimized conditions by one at the time method (individually change factors). Fig. 4 shows the suitable separation of Pen 6:3:3.

Fig. 4 shows the chromatogram of PG, BE and PR at 8 mM of TEA and 60 mM of TFA in phosphate buffer/MeOH, (55/45), flow rate of 1 ml min^{-1} at ambient temperature condition. The peaks at 2.820, 4.390 and 15.674 belong to the PR, BE and PG, respectively.

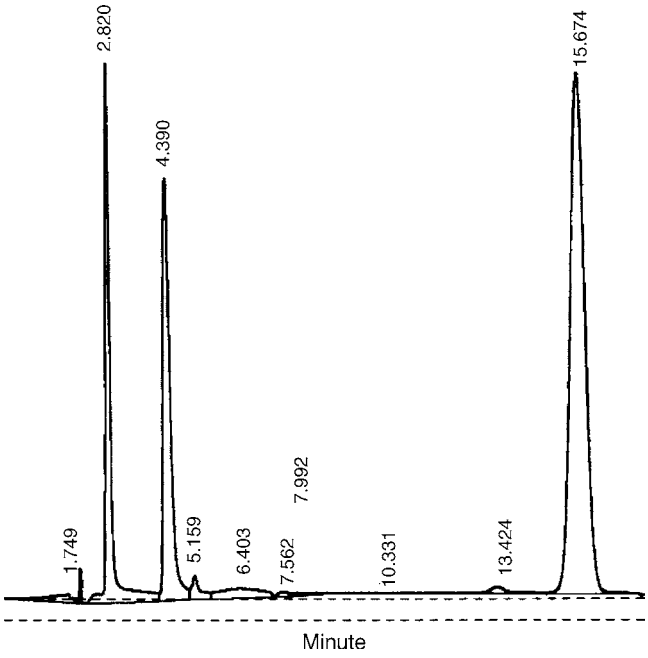

Fig. 4. The chromatogram of PG, BE and PR at 8 mM of TEA and 60 mM of TFA in phosphate buffer/MeOH, (55/45). Flow rate 1 of ml min^{-1} at ambient temperature. The peaks at 2.820, 4.390 and 15.674 belong to PR, BE and PG, respectively.

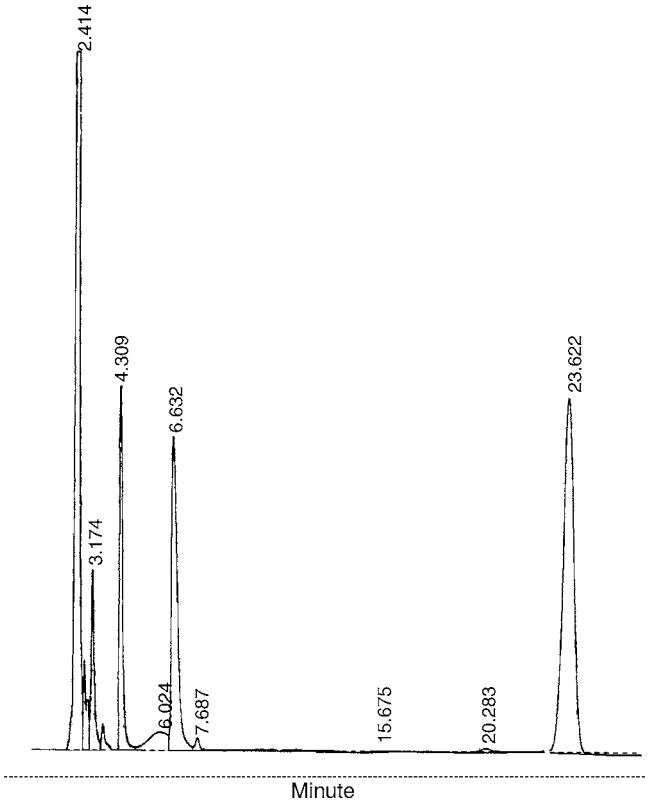

Fig. 5. The chromatogram of PG, PR and BE after extraction from serum. Conditions such as Fig. 4 but flow rate 0.8 ml min^{-1}. Peaks at 4.309, 6. 32 and 23.622 min belong to PR, BE and PG, respectively.

Fig. 4 shows that the presence of both TEA and TFA can improve the peak shape of these compounds.

Sensitivity of this method, defined as the slope of calibration curves of PG, PR and BE, was obtained from a set of seven points of calibration curves. The linear range was estimated using a best fit line.

The limit of detection (LOD, three-times the standard deviation of blank), linear concentration range, R.S.D. and calibration equation for PG are 1.1 $\mu g\,ml^{-1}$, 10–2400 $\mu g\,ml^{-1}$, 1.7% and $y = 0.0281x + 0.1273$ ($R^2 = 0.9999$); for BE: 1.2 $\mu g\,ml^{-1}$, 12–2100 $\mu g\,ml^{-1}$, 1.85 and $y = 0.0299x + 0.0056$ ($R^2 = 0.997$); for PR: 1.5 $\mu g\,ml^{-1}$, 20–2000 $\mu g\,ml^{-1}$, 2% and $y = 0.0234x + 0.0177$ ($R^2 = 0.997$), respectively.

The optimum condition was extended to the determination of Pen 6:3:3 in serum. In this case, the best resolution is obtained when the flow rate of the mobile phase is decreased to 0.8 ml min^{-1}. Fig. 5 shows the monitoring of Pen 6:3:3 in serum of patient after 6 h.

Fig. 5 represents the chromatogram of PG, PR and BE at optimum condition such as Fig. 4 but flow rate 0.8 ml min^{-1} in serum media, the peaks at 4.309, 6. 32 and 23.622 min belong to PR, BE and PG, respectively.

4. Conclusion

We have shown here that the reversed phase LC with polar hydro-organic mobile phase is a suitable method for separation of Pen 6:3:3 in drug formulation and serum. The ANOVA table of factorial design shows that variance ratio of TFA, TEA, MeOH, pH and flow rate are 22.885, 13.472, 4.398, 0.217 and 0.166, respectively.

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