Article

Experimental Design Approach for Development of HPLC Method for Simultaneous Analysis of Triamcinolone, Nystatin, and Gramicidin in Industrial Wastewater

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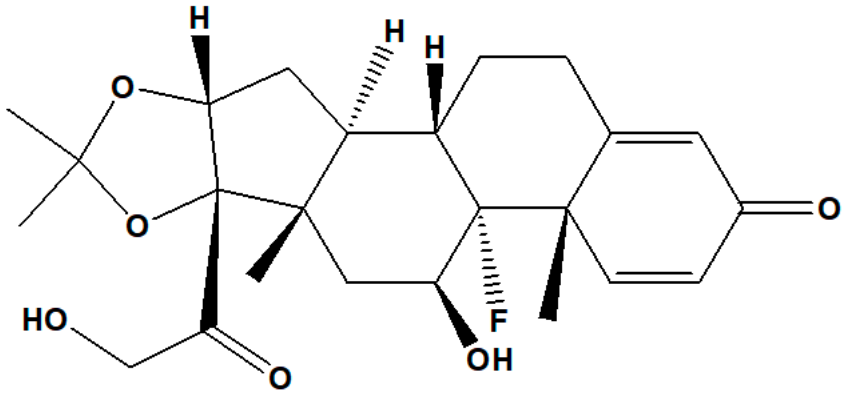
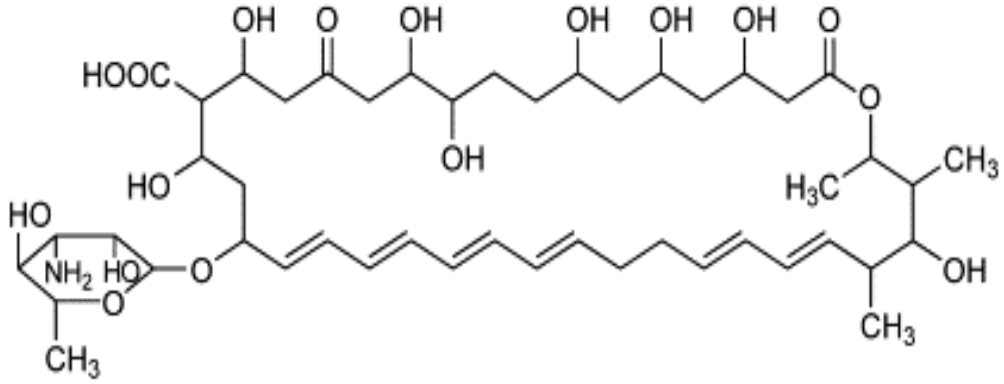


(a)



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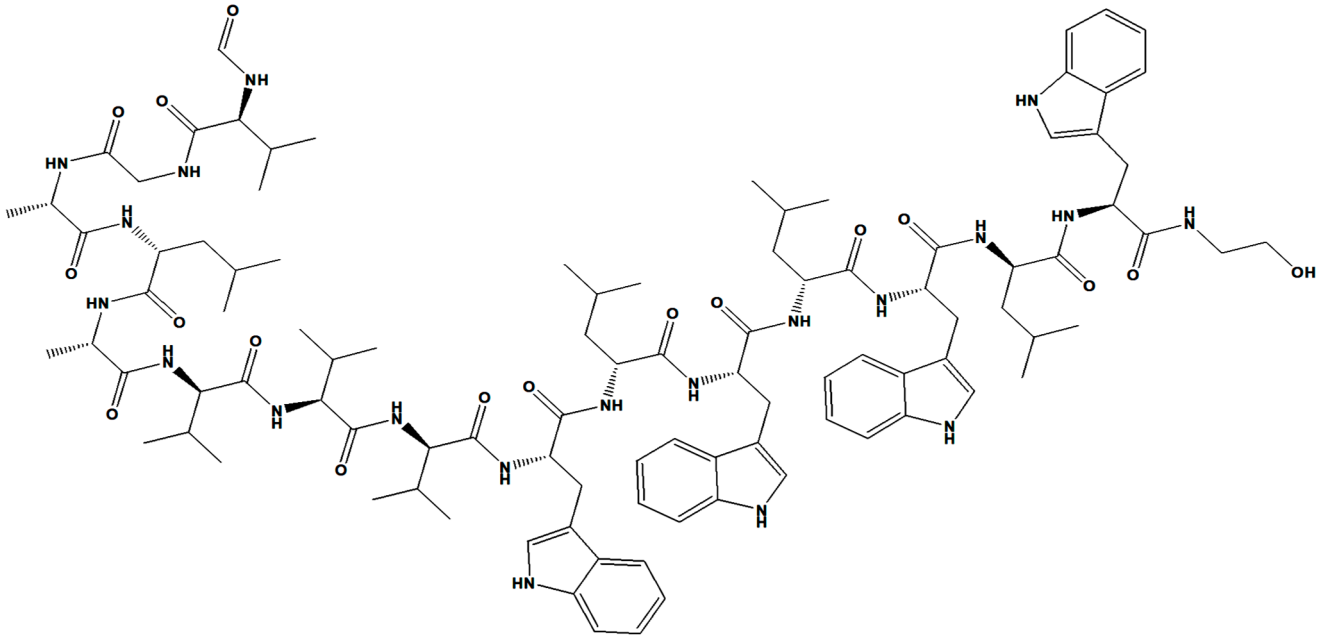

(c)

Figure S1: Chemical structures of triamcinolone (a), nystatin (b), and gramicidin (c)

Table S1. Coded and uncoded factors for the screening design for the purposed HPLC method

Coded	Uncoded		
	pH	**Organic Solvent %**	**Flow rate (mL/min)**
-1	2.50	85.00	1.00
0	3.00	87.00	1.20
+1	3.50	89.00	1.40

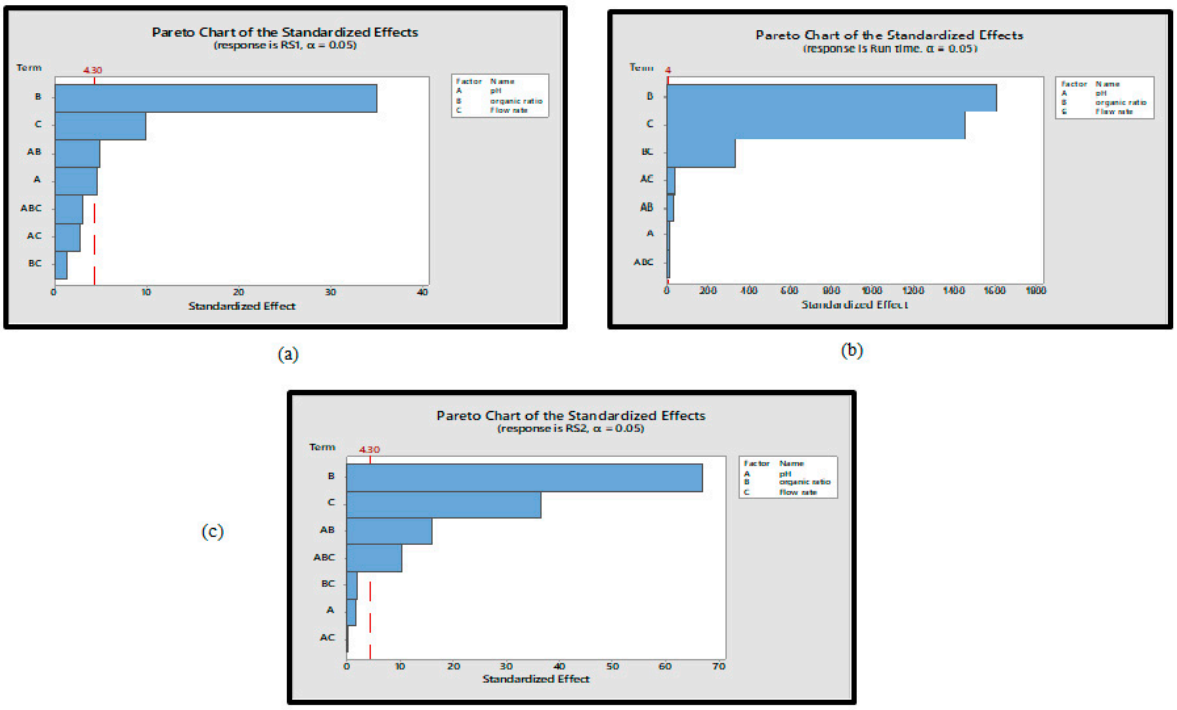

Figure S2. Pareto charts of the parameters and their effects on the response.

a: Effect of pH, organic ratio and flow rate on R_s1.

b: Effect of pH, organic ratio and flow rate on run time.

c: Effect of pH, organic ratio and flow rate on R_s2.

Table S2. Coded and uncoded factors for optimization design: Central Composite design for proposed HPLC method.

Coded	**Uncoded**		
	pH	**Organic Solvent %**	**Flow rate (mL/min)**
-1.68	2.2	83.6	0.9
-1	2.5	85.0	1.0
0	3.0	87.0	1.2
+1	3.5	89.0	1.4
+1.68	3.8	90.4	1.5

Table S3. A summary list ANOVA results in optimization design.

	P value of Model	R squared	R adjusted
R_s1	0.02	0.93	0.81
R_s2	0.05	0.71	0.68
Run time	0.00	0.98	0.96

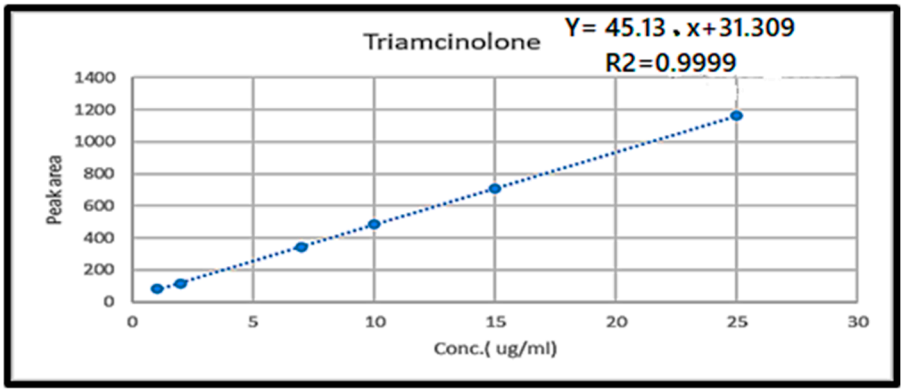

Figure S3. Calibration curve for triamcinolone (1.00-25.00 ug/mL).

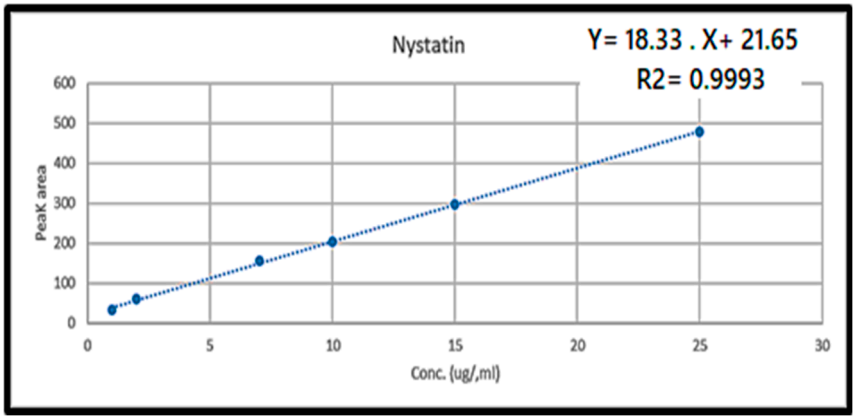

Figure S4. Calibration curve for nystatin (1.00-25.00 ug/mL).

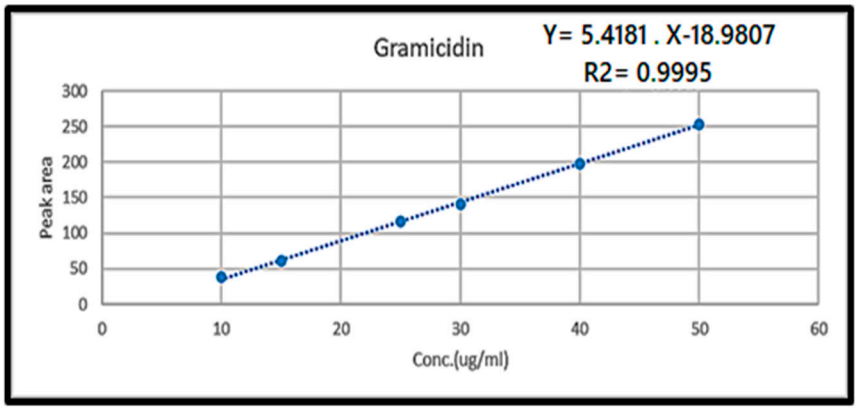

Figure S5. Calibration curve for gramicidin (10.00-50.00 ug/mL).

Table S4: Accuracy assessment of the proposed HPLC method for triamcinolone, nystatin and, gramicidin.

Triamcinolone			Nystatin			Gramicidin		
Amount taken (µg/ml)	**Amount found (µg/ml)**	**Recovery* %±SD**	**Amount taken (µg/ml)**	**Amount found**	**Recovery %**	**Amount taken (µg/ml)**	**Amount found**	**Recovery %**
5.00	4.99	99.80 ±0.01	5.00	4.95	99.08 ± 0.12	20.00	19.94	99.70 ±0.15
12.00	11.78	98.17 ±0.06	12.00	12.24	102.0 ± 0.17	35.00	35.69	101.97 ±0.22
22.00	21.83	99.23 ±0.06	22.00	22.43	101.95 ± 0.06	45.00	44.95	99.89 ±0.04
Mean Recovery % ±SD		99.07 ±0.04	101.01 ±0.12			100.52 ±0.14		

* Average of three determinations.

Table S5. Interday and intraday results for triamcinolone, nystatin and, gramicidin by the proposed HPLC method.

Triamcinolone			**Nystatin**			**Gramicidin**		
Intraday precision								
Amount taken (µg/mL)	**Amount found* (µg/mL)**	**% RSD**	**Amount taken (µg/mL)**	**Amount found (µg/mL)**	**% RSD**	**Amount taken (µg/mL)**	**Amount found (µg/mL)**	**% RSD**
5.00	4.994	0.15	**5.00**	5.10	1.26	**20.00**	20.10	0.50
12.00	11.766	0.49	**12.00**	12.30	1.41	**35.00**	34.90	0.62
22.00	21.77	0.28	**22.00**	22.20	0.26	**45.00**	45.23	0.09
Inter-day precision								
Amount taken (µg/mL)	**Amount found (µg/ml)**	**% RSD**	**Amount taken (µg/mL)**	**Amount found (µg/ml)**	**% RSD**	**Amount taken (µg/mL)**	**Amount found (µg/mL)**	**% RSD**
5.00	4.99	0.48	**5.00**	5.10	1.13	**20.00**	20.02	0.79
12.00	11.8	0.26	**12.00**	12.03	0.29	**35.00**	34.92	0.22
22.00	21.6	0.97	**22.00**	22.13	0.69	**45.00**	45.01	0.37

* Average of three determinations**.**

**%RSD: relative standard deviation percentage

Table S6. System Suitability parameters of the proposed HPLC method for determination of triamcinolone, nystatin, and gramicidin.

Parameters	Triamcinolone	Nystatin	Gramicidin	Reference value **[22]**
Resolution (R_s)	9.00	9.80		>1.5
Retention time (R_t; min)	2.90	3.70	5.60	-------
Selectivity (α)	2.91	1.77		α ≥1
Theoretical Plates (N)	3045	3539	3146	>2000
Tailing factor (T)	1.32	1.20	1.43	≤2
Capacity factor (k)	1.40	2.08	3.67	1-10

Table S7. Robustness design for the proposed HPLC method.

pH of phosphate buffer	Organic Solvent %	Flow rate (mL/min)	Rs1	Rs2	Run time
-1	1	-1	4.57±0.02	9.85±0.02	5.80±0.10
0	0	0	4.50±0.20	11.10±0.10	5.70±0.02
1	-1	1	4.20±0.30	12.80±0.25	5.60±0.02
-1	1	1	4.43±0.15	9.06±0.02	5.20±0.06
0	0	0	4.59±0.02	11.57±0.09	5.70±0.03
0	0	0	4.46±0.15	10.70±0.06	5.70±0.10
1	-1	-1	4.50±0.01	12.37±0.01	6.40±0.06
1	1	1	4.50±0.02	9.20±0.15	4.90±0.01
-1	-1	-1	4.67±0.07	11.10±0.11	6.50±0.01
-1	-1	1	4.70±0.06	10.30±0.10	5.90±0.02
1	1	-1	4.47±0.02	10.20±0.06	5.50±0.01

Table S8. Coded and the uncorded factors for the robustness design for HPLC method.

Coded	Uncoded		
	pH	**Organic Solvent %**	**Flow rate (mL/min)**
-1	3.5	86.7	1.2
0	3.6	87.7	1.3
+1	3.7	88.7	1.4