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In vitro study on interaction of ketotifen fumerate with amoxicillin trihydrate at different pH and are confirmed by IR spectroscopy

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The purpose of the present study was to investigate interaction between ketotifen fumerate and amoxicillin trihydrate in aqueous media of various pH (1.2, 2.8, 6.8 and 7.4). By using Job's continuous-variation analysis and Ardon's spectrophotomeric measurement methods, the values of stability constants of amoxicillin with ketotifen were determined at a fixed temperature (370C) at each of the medium pH. Stability constant, ranging between 0.0011 and 57.43, were derived from Ardon's plot, indicating that complexes formed, as a result of interaction between the drugs, were comparatively stable. However, when ketotifen is interacted with amoxicillin, stability constant were less than 1 at both gastric pH (1.2, 2.8) and intestinal pH (6.8 and 7.4) . But in vice versa when amoxicillin is interacted with ketotifen, stability constant values were more than 1 at gastric pH as well as at intestinal pH. Concurrent administration of ketotifen and amoxicillin trihydrate would result in the formation of a stable complex and this is likely to reduce the therapeutic activities of both drugs. There are many extra peaks observed in the IR spectra of interacted product of ketotifen fumarate with Amoxicillin trihydrate in the mixture ratio of 1:1 either in aqueous or chloroform extracts, in compared to that of their pure form.

Keyword: Stability Constant, Job's Method, Ardon's Mehod, Ketotifen Fumerate And Amoxicillin Trihydrate.

INTRODUCTION: Ketotifen is a benzocycloheptathiophene derivative that has been shown to possess anti-histaminic and anti-anaphylactic properties (Martin et al, 1977). It has been demonstrated that it can block in vitro release of mediators from rat peritoneal mast cells (Martin et al, 1977). The drug inhibits the release of histamine and leukotriene from basophil and lung tissue, antagonizes histamine at H1 receptors, inhibits calcium uptake, blocks passive cutaneous anaphylactic reaction, reverses isoprenaline-induced beta-adrenoceptor tachyphylaxis, and inhibits both allergen-induced and drug-induced asthma (Craps et al, 1978). A number of clinical trials of ketotifen have shown it to have a beneficial effect in the treatment of asthma (Hoshino et al, 1998 and Tinkelman et al, 1985) equivalent to that of disodium cromoglycate, which has an established place in the treatment of asthma (Clarke et al, 1980). Ketotifen, which is useful in the treatment of

hay fever and asthma, have been found to inhibit anaphylactic histamine release from animal tissues (Church et al, 1980). Amoxicillin (INN), formerly amoxycillin (BAN), and abbreviated amox, is a moderate-spectrum, bacteriolytic, β-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics. Amoxicillin is one of the most common antibiotics prescribed for children. Amoxicillin is used in the treatment of a number of infections including: acute otitis media, streptococcal pharyngitis, pneumonia, skin infections, urinary tract infections, salmonella, lyme disease, and chlamydia infections (The American Society of Health-System Pharmacists, Retrieved 3 April, 2011). It is used to prevent bacterial endocarditis in high risk people who are having dental work done, to prevent strep pneumococus infections in those without a spleen, and for both the prevention and the treatment of anthrax(The American Society of Health-System Pharmacists, Retrieved 3 April, 2011). It is also a treatment for cystic acne. The major goal of the present study was to elucidate the possible importance of drug-drug interactions (DDIs) as a contributing factor towards drug safety and finally to observe and determine the stability of the complexes, which could be formed between interaction of ketotifen fumarate and trihydrate amoxicillin in aqueous media of various pH. The values of stability constants of amoxicillin with ketotifen were determined by using Job's continuous-variation analysis and Ardon's spectrophotomeric measurement methods. However, when ketotifen is interacted with amoxicillin, the values of stability constants were 0.001 to 0.16 at both gastric pH (1.2, 2.8) and intestinal pH (6.8 and 7.4). But in vice versa when amoxicillin is interacted with ketotifen, stability constant values were between 3.39 and 57.43 at gastric pH as well as at intestinal pH.

Material and methods:

Drugs and chemicals:

Ketotifen fumerate and amoxicillin trihydrate were collected from Square Pharmaceuticals Ltd., Dhaka, Bangladesh as a token gift and were used without further purification. Sodium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate, used for the preparation of buffer solutions were purchased from Merck, Germany. Potassium chloride, sodium hydroxide, potassium hydroxide etc. were all of analytical grade.

Equipments:

UV-Visible spectrometer (Model No. UV-1600, Shimadzu, Japan), pH meter (Mettler Toledo, Switzerland), analytical balance (Model No. AL 204-S/01, Mettler Toledo, Switzerland), and a thermos tatted water bath (Shimadzu, Japan) were used for the test. A Dunbuff metabolic shakimg incubator (Nickel, Electrical Company, England) was used to shake the drug mixtures to attain equilibrium.

Preparation of standard solutions:

Stock solutions of ketotifen fumarate (1×10^{-3} M) and trihydrate amoxicillin (1×10^{-3} M) were prepared by dissolving them in distilled water. These stock solutions were diluted to desired strengths (1×10^{-5} M) by buffer solutions to obtain the working standard solutions.

Absorption spectrum analysis:

In observation of the spectra, the absorption characteristics of ketotifen fumerate and amoxicillin and their 1:1, 1:2 and 2:1 mixtures in the solutions of buffers (Perrin et al, 1974 and Mohiuddin et al, 2009) at pH 1.2, 2.8, 6.8 and 7.4 were compared with those of each interacting species. The concentrations of the sample were kept at very dilute levels in each case and the measurements made using UV-

VIS spectrophotometer with a constant temperature (25 ± 1) cell compartment and automatic recording unit. The stock solutions of the samples were diluted to appropriate levels, diluted with the buffers (1×10^{-5} M) at the desired pH and the spectra were recorded between 400 - 190 nm. The spectra were compared with those of the pure samples in each case.

Job's Spectrophotometric method:

According to Job's method (Job, 1928), a series of solutions were prepared in which the analytical concentration of one reactant (usually the cation) was held constant while that of the other was varied. Absorbance of series of ketotifen fumerate with amoxicillin in different molar ratios 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 were measured by keeping the total mole constant. The observed absorbance of the mixtures at various mole fractions was subtracted from sum of the values for free drugs (ketotifen fumerate and amoxicillin trihydrate). The absorbance difference (D) was then plotted against the mole fractions of the drug in the mixtures. If the formation constant is reasonably favorable, two straight lines of different slopes that intersect at a mole ratio that corresponds to the combining ratio in the complex are obtained (Sayeed et al, 2011).

Ardon's spectrometric method:

In the Ardon's spectrophotometric method, (Ardon, 1957) concentrations of ketotifen fumerate was varied while keeping the concentrations of other drugs fixed at 1×10^{-4} M. All the experiments were performed in buffer at pH 1.2, 2.8, 6.8 and 7.4. The absorbance of solutions having pH 1.2, 2.8, 6.8 and 7.4were measured at 300 nm using UV-VIS spectrophotometer. For calculations, the Ardon's equation was used. This equation is given below-

$$\frac{1}{(D-\epsilon_A C)} = \frac{1}{KC(\epsilon_{com}-\epsilon_A)[B]^n} + \frac{1}{C(\epsilon_{com}-\epsilon_A)}$$

Where,
D = Absorbance of the mixture.
B = Molar concentration of the Kitotifen fumerate.
C = Molar concentration of the other drug
ϵ_{com} = Molar extinction co-efficient of the complex.
ϵ_A = Molar extinction co-efficient of the Kitotifen fumerate.
The value of n was chosen as 1, which is an essential condition for validation of the method. The value for $1 / (D - \epsilon_A C)$ was plotted versus 1 / D to get the straight lines.
The stability constant of the complex was given by the relation, K = intercept / slope

It is to be mentioned that this method is only valid for the systems where 1:1 complexes are found (Sayeed et al, 2011).

Infra-red spectroscopy method:

The interaction between ketotifen fumarate and amoxicillin trihydrate were analyzed by IR spectrum analysis.100mg of pure powder of ketotifen fumarate was dissolved in 10ml of distilled water in a 50ml beaker, similarly 100mg of pure powder of Amoxicillin trihydrate was dissolved in another beaker. Both solutions were mixed in a separate 100ml beaker with constant stirring.Then the mixture was transferred to the separating funnel .The interacted drug was extracted in chloroform.Chloroform was evaporated and the precipitated solid drug product was analysed by IR spectrophotometer. Another experiment for aqueous extract was carried out by mixing both the drug solutions

together and evaporated. The drug product obtained was also analysed by IR spectrophotometer.IR spectrum of of pure powder of ketotifen fumarate and amoxicillin trihydrate was taken individually by using IR spectrophotometer.

Conc.of ketotifen	Absorbance(D value)			
	pH 1.2	pH 2.8	pH 6.8	pH 7.4
1.00E-05	0.184	0.223	0.195	0.65
2.00E-05	0.285	0.317	0.277	0.67
3.00E-05	0.356	0.391	0.35	0.667
4.00E-05	0.376	0.406	0.379	0.698
5.00E-05	0.376	0.434	0.377	0.705
6.00E-05	0.372	0.41	0.356	0.678
7.00E-05	0.352	0.363	0.355	0.625
8.00E-05	0.265	0.284	0.235	0.596
9.00E-05	0.17	0.187	0.166	0.572

TABLE 1 - Absorbance of ketotifen at different pH (using Job's method)

Results:

In spectral observation analysis, each of the drugs studied showed absorption in UV-VIS region. The molecular species of ketotifen fumerate and amoxicillin when separately mixed showed some changes in absorption characteristics of this drug molecule including some shifts in the absorption maxima. Initial detection of complexation of ketotifen fumerate with amoxicillin was done from the nature of spectra of pure compounds as well as their 1:1, 1:2 and 2:1 mixtures in buffer solution of pH 1.2, 2.8, 6.8 and 7.4 at a fixed concentration (1×10^{-5}) M. Continuous variation plot (Table 1 and Figure 3)gives information on the relative affinities of the complexes and it also depends on the intrinsic spectral characteristics of each complex.

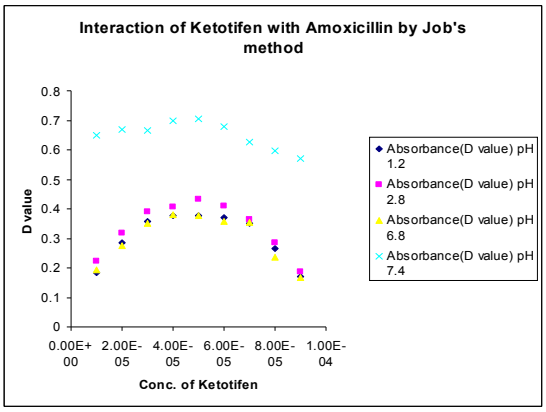


FIGURE 1 - Job's plot for complexation of ketotifen with amoxicillin at 300 nm.

The numeric values of the resulting stability constants were between 1.57 and 2.12 when complexation occurs among the amoxicillin and ketotifen (Table 5). There are many extra peaks observed in the IR spectra of interacted product of ketotifen fumarate with Amoxicillin trihydrate in the mixture ratio of 1:1 either in aqueous or chloroform extracts, in compared to that of their pure form. The presence of the extra peaks confirmed that there is a interaction between Ketotifen fumarate and Amoxicillin trihydrate (Table 6)

$1/D \times 10^{-5}$	$1/(D-CC_A)$			
	pH=1.2	pH= 2.8	pH= 6.8	pH= 7.4
1	41.667	47.619	62.5	76.923
0.5	13.699	41.667	23.256	43.478
0.33	6.579	32.258	8.85	16.667
0.25	4.386	8.264	4.717	12.987
0.2	2.907	4.545	3.03	12.346
0.167	2.041	2.618	2.132	5.993
0.143	1.468	1.862	1.497	5.076

TABLE 2 - Absorbance of ketotifen at different pH (using Ardon's method, when conc. of amoxicillin is constant)

Discussions:

It is obvious that each compound has its unique molecular structure or electronic configuration which is responsible for absorption of light in the form of ultraviolet or visible form. For this reason the spectrum of any pure compound obtained from UV spectrum will be of one kind that will be totally different from the other compound or the complex of that compound with other compounds. It spectra of alone ketotifen fumerate at different pH showed a sharp absorption maximum at 300 nm. When amoxicillin mixed with ketotifen in 2:1 ratio the intensity of the peak of ketotifen change remarkably (absorbance decreases) i.e. absorption characteristics are altered due to interaction but the position of the compound do not shift.

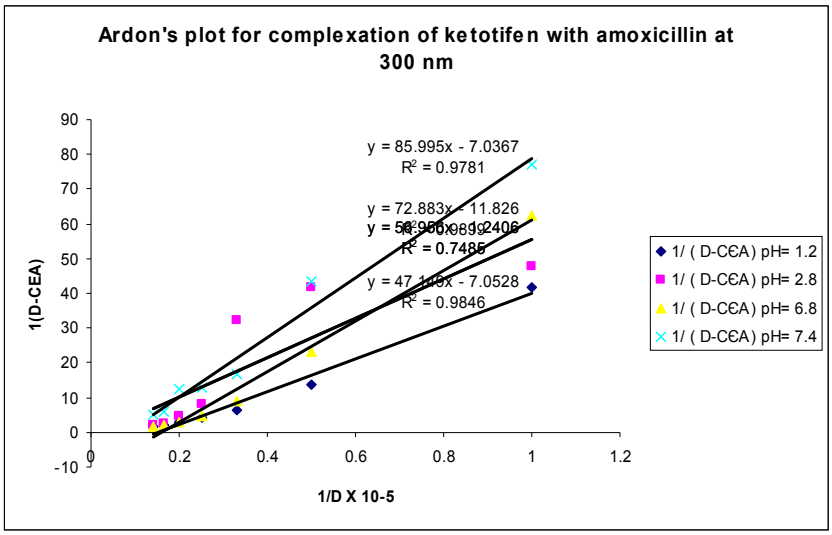


FIGURE 2 - Ardon's plot for complexation of ketotifen with amoxicillin at 300 nm.

TABLE 3 - Stability constant of ketotifen with amoxicillin at different pHs

System	pH	Stability Constants($1X10^{-2}$)
Interaction of ketotifen with amoxicillin	pH=1.2	0.11
	pH=2.8	2.18
	pH= 6.8	16.23
	pH= 7.4	8.18

TABLE 4 - Absorbance of ketotifen at different pH (using Ardon's method, when concentration of ketotifen is constant)

$1/D \times 10^{-5}$	$1/(D-C\epsilon_A)$			
	pH=1.2	pH= 2.8	pH= 6.8	pH= 7.4
1	31.25	10.309	6.452	8.547
0.5	47.619	7.042	6.061	8
0.33	21.277	12.195	5.814	9.346
0.25	25.641	9.346	5.587	-
0.2	52.632	7.752	5.464	9.524
0.167	31.25	10.87	5.155	9.259
0.143	20.833	12.987	5.051	7.042

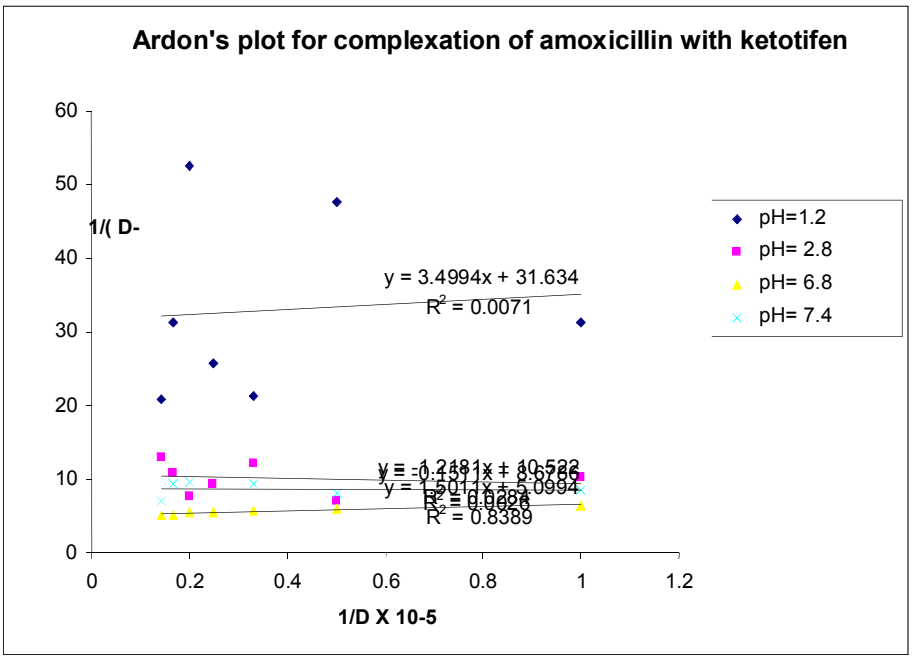


FIGURE 3 - Ardon's plot for complexation of amoxicillin with ketotifen at 300 nm.

TABLE 5 - Stability constant of amoxicillin with ketotifen at different pH

System	pH	Stability Constants
Interaction of ketotifen with amoxicillin	pH=1.2	9.04
	pH=2.8	8.64
	pH= 6.8	3.39
	pH= 7.4	57.43

Table: 6 Interacted peaks for ketotifen fumarate and amoxicillin trihydrate

Aqueous extract Wave No. (cm^{-1})	Chloroform extract Wave No. (cm^{-1})
703.08	840.04
1595.2	1038.71
2311.79	1362.77
2625.23	1559.51
2729.39	1635.71
2954.11	2311.79
3014.87	2603.05
3114.21	2749.65
3210.65	3012.94
3357.25	3198.11

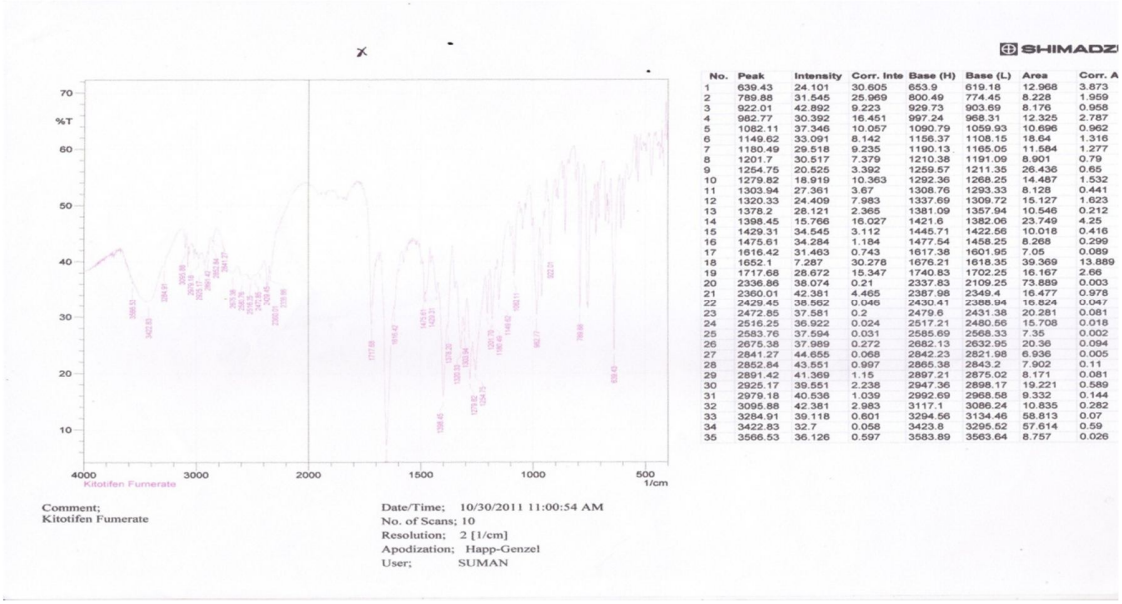
The Ardon's plots have been used to evaluate the stability constants and it has been observed that when values of 1 / (D - $C_{\varepsilon A}$) are plotted against 1/ Drug (Figure 4 and 5), good straight lines are obtained obeying the Ardon's equation. The value of stability constants at different pH are shown in table 3 and 5. Very low stability constant numeric values (values less than 1) mean that the formation of complex due to interaction among the drugs is readily dissociated (Table 3), yielding essentially all drugs in ionic form at pH as low as stomach acid (about pH 2 to 3) to as high as physiologic pH 7.4 (the pH of the main extracellular body fluids such as serum and lymph).These values are the signs and symptoms of good interaction between ketotifen with amoxicillin. It can be assumed that these two drugs cannot safely be administered orally at a time. Following the Ardon,s method when amoxicillin is considered as the parent drug and interacted with ketotifen a lower stability constant values were found which indicate the readily solubility of both drugs and minimum drug-drug interaction (Figure 4). There are as many peaks observed in the IR spectrum of combination of the two drugs Ketotifen Fumarate and Amoxicillin trihydrate in both aqueous and chloroformic extracts as compared to that of pure their pure forms. From these extra peaks we confirm that there is an interaction between Ketotifen Fumarate and Amoxicillin trihydrate in combination form(Table 6)

Conclusion:

Interaction of ketotifen with amoxicillin decreased the free drug concentration of both drugs which can result in decreased availability of the drugs at receptors. Ultimately, one or both drugs may show diminished pharmacologic activity. Furthermore, ketotifen fumarate and amoxicillin lowered protein binding of amoxicillin could increase the volume of distribution of amoxicillin. Therefore, cautions should be exercised during administration of both drugs, pending *in vivo* experiments to determinethe implication of our findings. From the invivo and IR study it is confirmed that there are interaction between Ketotifen fumarate and Amoxicillin trihydrate when administered concurrently. Therefore cautions must be taken when these combination drugs are administered to minimize the risk of drug interaction and get maximum therapeutic efficacy of the individual drugs to cure the illness of the patient in its rational use.Further studies can be carried out to determine whether these drug interactions are beneficial or harmful.

Infrared Study

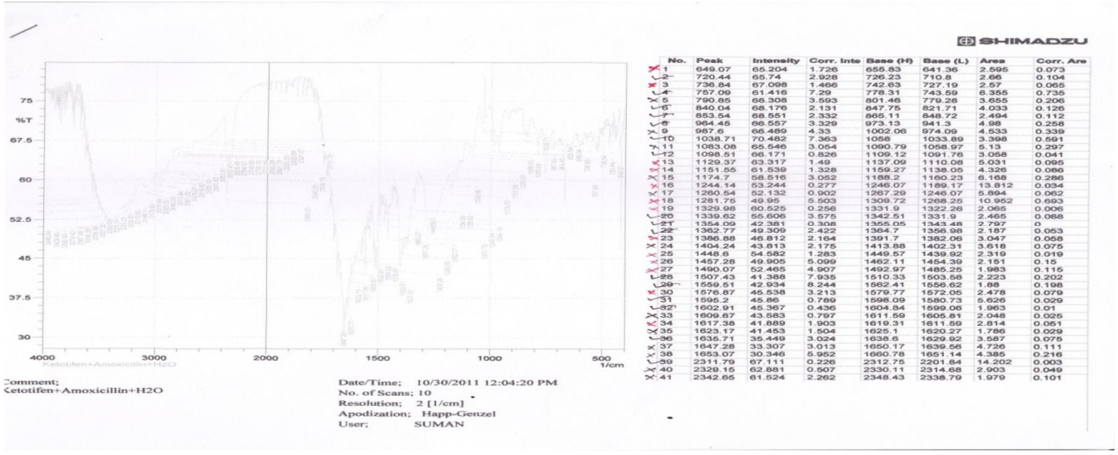
IR Spectrum of Ketotifen Fumarate



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. A
1	639.43	24.101	30.605	653.9	619.18	12.968	3.873
2	789.88	31.545	25.969	800.49	774.45	8.228	1.959
3	922.01	42.892	9.223	929.73	903.69	8.176	0.958
4	982.77	30.392	16.451	997.24	968.31	12.325	2.787
5	1082.11	37.346	10.057	1090.79	1059.93	10.696	0.962
6	1149.62	33.091	8.142	1156.37	1108.15	18.64	1.316
7	1180.49	29.518	9.235	1190.13	1165.05	11.584	1.277
8	1201.7	30.517	7.379	1210.38	1191.09	8.901	0.79
9	1254.75	20.525	3.392	1259.57	1211.35	26.436	0.65
10	1279.82	18.919	10.363	1292.36	1268.25	14.487	1.532
11	1303.94	27.361	3.67	1308.76	1293.33	8.128	0.441
12	1320.33	24.409	7.983	1337.69	1309.72	15.127	1.623
13	1378.2	28.121	2.365	1381.09	1357.94	10.546	0.212
14	1398.45	15.766	16.027	1421.6	1382.06	23.749	4.25
15	1429.31	34.545	3.112	1445.71	1422.56	10.018	0.416
16	1475.61	34.284	1.184	1477.54	1458.25	8.268	0.299
17	1616.42	31.463	0.743	1617.38	1601.95	7.05	0.089
18	1652.1	7.287	30.278	1676.21	1618.35	39.369	13.889
19	1717.68	28.672	15.347	1740.83	1702.25	16.167	2.66
20	2336.86	38.074	0.21	2337.83	2109.25	73.889	0.003
21	2360.01	42.381	4.465	2387.98	2349.4	16.477	0.978
22	2429.45	38.562	0.046	2430.41	2388.94	16.824	0.047
23	2472.85	37.581	0.2	2479.6	2431.38	20.281	0.081
24	2516.25	36.922	0.024	2517.21	2480.56	15.708	0.018
25	2583.76	37.594	0.031	2585.69	2568.33	7.35	0.002
26	2675.38	37.989	0.272	2682.13	2632.95	20.36	0.094
27	2841.27	44.655	0.068	2842.23	2821.98	6.936	0.005
28	2852.84	43.551	0.997	2865.38	2843.2	7.902	0.11
29	2891.42	41.369	1.15	2897.21	2875.02	8.171	0.081
30	2925.17	39.551	2.238	2947.36	2898.17	19.221	0.589
31	2979.18	40.536	1.039	2992.69	2968.58	9.332	0.144
32	3095.88	42.381	2.983	3117.1	3086.24	10.835	0.282
33	3284.91	39.118	0.601	3294.56	3134.46	58.813	0.07
34	3422.83	32.7	0.058	3423.8	3295.52	57.614	0.59
35	3566.53	36.126	0.597	3583.89	3563.64	8.757	0.026

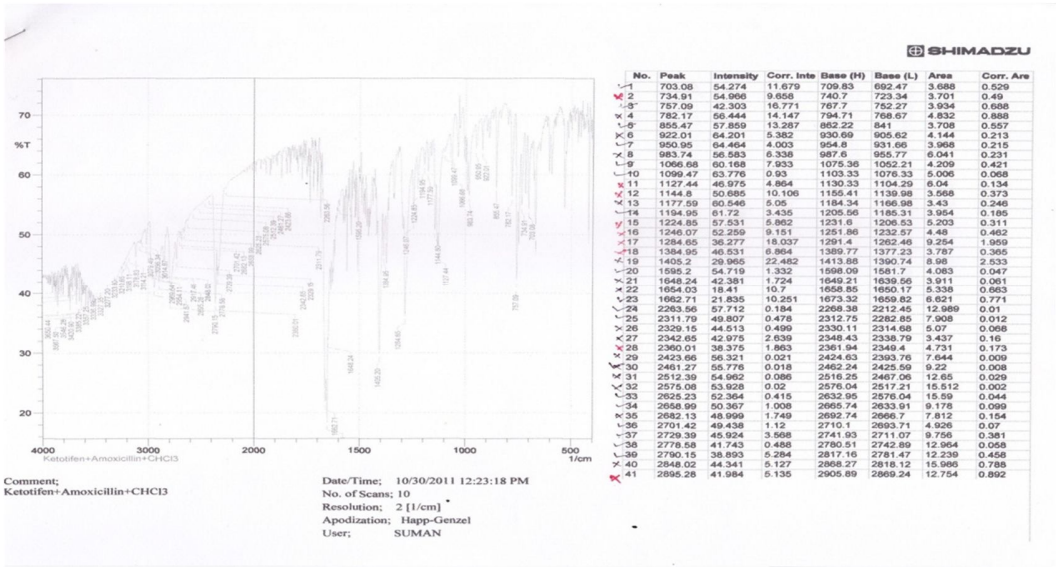
IR Spectrum Of Amoxicillin Trihydrate

IR Spectrum Of Ketotifen Fumarate+Amoxicillin Trihydrate (Aqueous Extract)



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	649.07	65.204	1.726	655.83	641.36	2.595	0.073
2	720.44	65.74	2.928	726.23	710.8	2.66	0.104
3	736.84	67.098	1.466	742.63	727.19	2.57	0.065
4	757.09	61.419	7.29	778.31	743.59	6.355	0.735
5	790.85	66.308	3.593	801.46	779.28	3.685	0.206
6	840.04	68.176	2.131	847.75	821.71	4.033	0.126
7	853.54	68.551	2.332	865.11	848.72	2.494	0.112
8	964.48	66.557	3.329	973.13	941.3	4.98	0.258
9	987.6	66.469	4.33	1002.06	974.09	4.533	0.339
10	1038.71	70.482	7.363	1058	1033.89	3.398	0.591
11	1083.08	65.546	3.054	1090.79	1058.97	5.13	0.297
12	1098.51	66.171	0.826	1109.12	1091.76	3.058	0.041
13	1129.37	63.317	1.49	1137.09	1110.08	5.031	0.095
14	1151.55	61.539	1.328	1159.27	1138.05	4.326	0.086
15	1174.7	58.516	3.082	1188.2	1160.23	6.168	0.286
16	1244.14	53.244	0.277	1246.07	1189.17	13.812	0.034
17	1260.54	52.132	0.902	1267.29	1246.07	5.894	0.062
18	1281.75	49.95	5.503	1309.72	1268.25	10.952	0.693
19	1329.98	60.525	0.256	1331.9	1322.26	2.065	0.006
20	1339.62	56.606	3.575	1342.51	1331.9	2.465	0.088
21	1354.09	42.381	0.308	1355.05	1343.48	2.797	0
22	1362.77	49.309	2.422	1364.7	1356.98	2.187	0.053
23	1386.88	46.812	2.164	1391.7	1382.06	3.047	0.058
24	1404.24	43.813	2.175	1413.88	1402.31	3.518	0.075
25	1448.6	54.582	1.283	1449.57	1439.92	2.319	0.019
26	1457.28	49.905	5.099	1462.11	1454.39	2.181	0.15
27	1490.07	52.465	4.907	1492.97	1485.25	1.983	0.115
28	1507.43	41.388	7.935	1510.33	1503.58	2.223	0.202
29	1559.51	42.934	8.244	1562.41	1556.62	1.88	0.198
30	1576.87	45.538	3.213	1579.77	1572.05	2.478	0.079
31	1596.2	45.86	0.789	1598.09	1580.73	5.626	0.029
32	1609.87	43.583	0.797	1611.59	1605.81	2.048	0.025
33	1617.38	41.889	1.903	1619.31	1611.59	2.814	0.051
34	1623.17	41.453	1.504	1625.1	1620.27	1.786	0.029
35	1636.71	35.449	3.024	1638.6	1629.92	3.587	0.075
36	1647.28	33.307	3.013	1650.17	1639.56	4.726	0.111
37	1653.07	30.346	5.952	1660.78	1651.14	4.385	0.216
38	2311.79	67.111	0.226	2312.75	2201.84	14.202	0.003
39	2329.15	62.881	0.507	2330.11	2314.68	2.903	0.049
40	2342.65	61.524	2.262	2348.43	2338.79	1.979	0.101
41							

IR Spectrum of Ketotifen Fumarate + Amoxicillin Trihydrate (Chloroform Extract)



No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	703.08	54.274	11.679	709.83	692.47	3.688	0.529
2	734.91	54.966	9.658	740.7	723.34	3.701	0.49
3	757.09	42.303	16.771	767.7	752.27	3.934	0.688
4	782.17	56.444	14.147	794.71	768.57	4.832	0.888
5	855.47	57.859	13.287	862.22	841	3.708	0.557
6	922.01	64.201	5.382	930.69	905.62	4.144	0.213
7	950.95	64.464	4.003	954.8	931.66	3.968	0.215
8	983.74	56.583	6.338	987.6	955.77	6.041	0.231
9	1066.68	60.168	7.933	1075.36	1052.21	4.209	0.421
10	1099.47	63.776	0.93	1103.33	1076.33	5.006	0.068
11	1127.44	46.975	4.864	1130.33	1104.29	6.04	0.134
12	1144.8	50.685	10.106	1155.41	1139.98	3.568	0.373
13	1177.59	60.546	5.05	1184.34	1166.98	3.43	0.246
14	1194.96	61.72	3.435	1205.56	1185.31	3.954	0.186
15	1224.85	57.531	5.862	1231.6	1206.53	5.203	0.311
16	1246.07	52.259	9.151	1251.86	1232.57	4.48	0.462
17	1284.65	36.277	18.037	1291.4	1262.46	9.254	1.959
18	1384.95	46.531	6.864	1389.77	1377.23	3.787	0.365
19	1405.2	29.965	22.482	1413.86	1390.74	8.98	2.533
20	1595.2	54.719	1.332	1598.09	1581.7	4.083	0.047
21	1648.24	42.381	1.724	1649.21	1639.56	3.911	0.061
22	1654.03	18.41	10.7	1658.85	1650.17	5.338	0.663
23	1662.71	21.835	10.251	1673.32	1659.82	6.621	0.771
24	2263.56	57.712	0.184	2268.38	2212.45	12.989	0.01
25	2311.79	49.807	0.476	2312.75	2282.85	7.908	0.012
26	2329.15	44.513	0.499	2330.11	2314.68	5.07	0.068
27	2342.65	42.975	2.639	2348.43	2338.79	3.437	0.16
28	2360.01	38.375	1.863	2361.94	2349.4	4.731	0.173
29	2423.66	56.321	0.021	2424.63	2393.76	7.644	0.009
30	2461.27	55.776	0.018	2462.24	2425.59	9.22	0.008
31	2512.39	54.962	0.086	2516.25	2467.06	12.65	0.029
32	2575.08	53.928	0.02	2576.04	2517.21	15.512	0.002
33	2625.23	52.364	0.415	2632.95	2576.04	15.59	0.044
34	2658.99	50.367	1.008	2665.74	2633.91	9.178	0.099
35	2682.13	48.999	1.749	2692.74	2666.7	7.812	0.154
36	2701.42	49.438	1.12	2710.1	2693.71	4.926	0.07
37	2729.39	45.924	3.568	2741.93	2711.07	9.756	0.381
38	2778.58	41.743	0.488	2780.51	2742.89	12.964	0.058
39	2790.15	38.893	5.284	2817.16	2781.47	12.239	0.458
40	2848.02	44.341	5.127	2868.27	2818.12	15.986	0.788
41	2895.28	41.984	5.135	2905.89	2869.24	12.754	0.892

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