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Research

Development of Validated Analytical Methods for Levosimendan and its Application in *in-vitro* Interaction Studies

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Abstract:
This study outlines the development and validation of multiple analytical methods for the quantification of levosimendan in injectable formulations, including UV spectrophotometry, spectrofluorimetry, high-performance thin-layer chromatography (HPTLC), and stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC). Each method was optimized for sensitivity, specificity, and reproducibility, and successfully validated in accordance with ICH guidelines. The UV spectrophotometric method showed excellent linearity in the range of 1–5 µg/mL with a correlation coefficient (R^2) of 0.9997 and λmax at 401 nm. Spectrofluorimetric analysis, based on the oxidation of levosimendan with ceric ammonium sulphate, demonstrated a sensitive response in the range of 400–2000 ng/mL with mean recoveries exceeding 98%. For HPTLC, levosimendan was effectively separated using a mobile phase of THF:DCM:ether (1:8:1, v/v), showing a sharp peak at Rf 0.74 and high accuracy and precision across a 200–1000 ng/band concentration range. The stability-indicating RP-HPLC method utilized a C18 column with a mobile phase of ammonium acetate buffer and methanol (48:52, v/v), achieving optimal separation with a retention time of ~11 min and excellent resolution, linearity (0.2–1.2 µg/mL), and robustness under various stress conditions. The validated RP-HPLC method was applied in in-vitro drug interaction studies with aspirin, clopidogrel, and atorvastatin using equilibrium dialysis. Results indicated significant displacement of levosimendan from protein binding sites, particularly with atorvastatin (14.74%) and aspirin (7.77%), suggesting potential pharmacokinetic interactions. Overall, the developed methods proved to be reliable, accurate, and robust for the quantitative analysis of levosimendan in injectable dosage forms, with the RP-HPLC method also demonstrating suitability for in-vitro interaction and stability studies.

Keywords: Levosimendan, UV Spectrophotometry, Spectrofluorimetry, HPTLC, RP-HPLC, Method Validation, In-vitro Drug Interaction, Stability-Indicating Method

1. Introduction:

Levosimendan is a calcium sensitizer and potassium channel opener that has been widely utilized in the treatment of acute decompensated heart failure due to its

dual inotropic and vasodilatory effects. It enhances myocardial contractility without increasing intracellular calcium levels, thereby reducing the risk of arrhythmias. Owing to its unique pharmacological profile, levosimendan has gained significant interest in both clinical and research settings.

For effective therapeutic monitoring and quality control of pharmaceutical formulations containing levosimendan, the development of robust, sensitive, and accurate analytical methods is essential. The reliable quantification of levosimendan not only supports pharmaceutical quality assurance but also facilitates pharmacokinetic and drug interaction studies.

Various analytical techniques have been employed for drug estimation, each offering specific advantages in terms of sensitivity, specificity, and applicability. However, limited comprehensive studies are available that validate multiple analytical techniques for levosimendan in injectable formulations. Moreover, considering the increasing trend of polypharmacy in cardiovascular conditions, the study of potential drug-drug interactions of levosimendan with commonly co-administered agents such as aspirin, clopidogrel, and atorvastatin becomes critical.

This study aims to develop and validate UV spectrophotometric, spectrofluorimetric, HPTLC, and RP-HPLC methods for the determination of levosimendan in injection dosage forms. Furthermore, the RP-HPLC method is employed for stability studies under various stress conditions and in-vitro interaction analysis with selected cardiovascular drugs. The proposed analytical approaches are expected to aid in the routine analysis of levosimendan and enhance understanding of its pharmacokinetic interactions, thereby contributing to improved therapeutic management.

2. Materials and Methods

2.1 Chemicals and Reagents

Levosimendan standard was obtained from a certified supplier. All reagents used were of analytical or HPLC grade. Methanol, tetrahydrofuran (THF), dichloromethane (DCM), diethyl ether, ammonium acetate, ceric ammonium sulphate (CAS), sulphuric acid, disodium hydrogen phosphate, acetonitrile (ACN), and hydrogen peroxide (15%) were purchased from reputable vendors. Bovine serum albumin (BSA) was used for protein binding studies. Deionized water was used throughout the experimental procedures.

2.2 Instrumentation

A UV-visible spectrophotometer was used to record absorbance spectra. Spectrofluorimetric measurements were performed using a calibrated spectrofluorimeter. High-performance thin-layer chromatography (HPTLC) analysis was carried out on silica gel 60 F254 plates using a CAMAG HPTLC system. Reverse-phase high-performance liquid chromatography (RP-HPLC) was conducted using a system equipped with a C18 column and UV detector. Data acquisition and processing were performed using appropriate chromatographic software.

2.3 Development and Validation of Analytical Methods

2.3.1 UV Spectrophotometric Method

Levosimendan was found to be freely soluble in methanol and all stock and working standard solutions were prepared in methanol. The absorbance spectrum of levosimendan was scanned in the UV range, with a maximum absorbance (λ_max) observed at 401 nm. Linearity was established in the range of 1–5 µg/mL, with a correlation coefficient (R^2) of 0.9997. The method was validated as per ICH guidelines, and sensitivity was demonstrated with LOD and LOQ values of 0.0749 µg/mL and 0.2272 µg/mL, respectively. Recovery studies confirmed the accuracy of the method, while intra-day and inter-day precision studies (RSD < 2%) established its repeatability. Specificity was confirmed in the presence of formulation excipients.

2.3.2 Spectrofluorimetric Method

This method was based on the oxidation of levosimendan by ceric ammonium sulphate (CAS), producing fluorescent cerium (III) species. The reaction was carried out in 0.05 M sulphuric acid, followed by heating at 50 °C for 20 min. Methanol was used as the initial solvent for levosimendan, while water was used for dilution post-reaction to enhance fluorescence intensity. The fluorescence response was linear over the concentration range of 400–2000 ng/mL. Validation parameters confirmed the method's accuracy (recovery >98%), precision (repeatability within acceptable limits), and specificity (no excipient interference). LOD and LOQ values supported the method's sensitivity.

2.3.3 HPTLC Method

Chromatographic separation was performed using silica gel 60 F254 plates and a mobile phase consisting of THF:DCM:diethyl ether (1:8:1, v/v). The plates were developed and visualized in an iodine chamber before scanning at 401 nm. A compact, well-resolved peak of levosimendan was observed at an Rf value of 0.74. Linearity was established between 200–1000 ng/band (R^2 = 0.9937). Method validation included recovery studies (99.83–100.09%), repeatability, precision (%RSD <2), and specificity. Stability studies confirmed the robustness of the method.

2.3.4 RP-HPLC Method

Method development employed a C18 column with a mobile phase of ammonium acetate buffer and methanol (48:52, v/v) at a flow rate optimized to yield symmetric peaks with minimal tailing. A retention time of ~11.4 min was observed for levosimendan. Calibration was linear over 0.2–1.2 µg/mL. Accuracy was confirmed via recovery studies, and precision (intra-day and inter-day %RSD <2%) and robustness testing supported method reliability. Stability assessments indicated levosimendan remained stable under analytical conditions.

2.4 Forced Degradation Studies

Stability-indicating capability of the RP-HPLC method was assessed under stress conditions including acid (0.01 M HCl), base (0.01 M NaOH), neutral (water), oxidative (15% H_2O_2), and photolytic degradation (UV exposure). Samples were analyzed immediately and after 2 hours of stress exposure. Chromatographic analysis showed selective and resolved peaks of levosimendan, with no co-eluting degradants, confirming the method's specificity and stability-indicating nature.

2.5 In-vitro Drug Interaction and Protein Binding Studies

In-vitro interaction studies of levosimendan with co-administered drugs (aspirin, clopidogrel, atorvastatin) were conducted using the validated RP-HPLC method. Equilibrium dialysis was employed to evaluate protein binding with BSA. Drug concentrations were fixed at 2×10^{-5} M, and levosimendan was found to have 79.69% protein binding under baseline conditions. The presence of aspirin, clopidogrel, and atorvastatin displaced levosimendan from BSA by 7.77%, 3.94%, and 14.74%, respectively. Chromatographic separation demonstrated no interference between peaks of levosimendan and interacting drugs. Levosimendan was stable in phosphate buffer (pH 7.4) for 48 h, confirming its suitability for protein binding assays.

3. Results and Discussion

1. UV Spectrophotometric Method

The developed UV spectrophotometric method for levosimendan exhibited excellent sensitivity and linearity within the concentration range of 1–5 µg/mL, with a correlation coefficient (r^2) of 0.9997, indicating a strong linear relationship. The method demonstrated good sensitivity with a limit of detection (LOD) and limit of quantification (LOQ) of 0.0749 µg/mL and 0.2272 µg/mL, respectively. The accuracy was confirmed by recovery studies with values nearing 100%, while the precision was supported by low intra- and inter-day variability. The assay of the marketed formulation yielded a mean concentration of 12.3987 mg, aligning closely with the label claim, thereby confirming the method's suitability for routine quality control of levosimendan injections.

2. Spectrofluorimetric Method

The spectrofluorimetric method developed based on cerium (III) fluorescence formation provided an alternative, highly sensitive approach. A linear response was observed in the range of 400–2000 ng/mL, with recovery values above 98%, confirming the method's accuracy. Precision and repeatability were validated by low standard deviation and %RSD values. The LOD and LOQ values supported the method's sensitivity, while specificity was demonstrated by a lack of interference from excipients. The assay of a commercial formulation yielded a satisfactory label claim of 99.08%, validating the method's application in pharmaceutical analysis.

3. HPTLC Method

The optimized HPTLC method using a THF:DCM:ether (1:8:1 v/v) mobile phase on silica gel 60F254 plates produced compact and well-resolved spots with an Rf value of 0.74. The method exhibited linearity between 200–1000 ng/band (r^2 = 0.9937). Accuracy was confirmed with recovery values between 99.83%–100.09%, and precision was validated through consistent intra- and inter-day results (%RSD <2). The assay results of levosimendan injection (99.69%) confirmed the method's reliability and applicability for routine quantitative analysis.

4. RP-HPLC Method and Stability Studies

4.1 Assay and Method Validation

The stability-indicating RP-HPLC method developed using a C18 column with ammonium

acetate:methanol (48:52 v/v) as the mobile phase yielded sharp, symmetrical peaks with acceptable tailing factor (<1.5). Linearity was observed in the range of 0.2–1.2 µg/mL with a strong correlation coefficient. The method exhibited high recovery, precision (%RSD <2), and robustness under varied conditions. The mean assay result (12.4394 mg) corresponded well with the product label, confirming the method's accuracy and reproducibility.

4.2 Forced Degradation Studies

Stress testing confirmed the method's stability-indicating nature. Acid hydrolysis resulted in 37.29% degradation, while base and oxidative conditions led to moderate to complete degradation over time. Neutral hydrolysis produced additional peaks, indicating degradation products. Photolytic stress resulted in minimal degradation (9.33%). All degraded samples showed clear separation and acceptable peak purity, confirming the method's specificity and robustness.

5. In-Vitro Drug Interaction Studies

Simultaneous separation of levosimendan with aspirin, clopidogrel, and atorvastatin using the RP-HPLC method demonstrated satisfactory resolution and no significant interference. Protein binding interaction studies using equilibrium dialysis revealed significant displacement of levosimendan from BSA upon co-incubation with aspirin (7.77%) and atorvastatin (14.74%), while clopidogrel induced moderate displacement (3.94%). These results suggest that co-administration of levosimendan with these agents may influence its free plasma concentration and therapeutic activity, particularly with atorvastatin.

Table 1: Calibration data for levosimendan by UV spectrophotometric method

S. No	Concentration (µg/ml)	Absorbance
1	1	0.1559
2	2	0.3153
3	3	0.4584
4	4	0.6110
5	5	0.7505

Fig. 3: Linearity graph of levosimendan

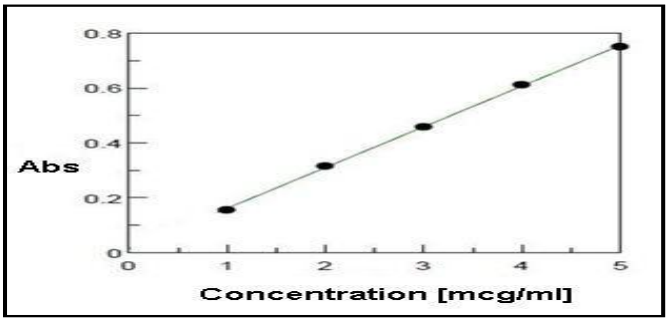

Table 2: Recovery data of levosimendan by UV method

Conc. of drug taken (µg/ml)	Conc. of standard added (µg/ml)	Mean Conc. found ± SD*	Mean Recovery (%)	% RSD*
3	1.5 (50%)	4.4891±0.0108	99.76	0.2406
3	3 (100%)	6.025±0.0138	100.42	0.2290
3	4.5 (150%)	7.4562±0.0394	99.41	0.2602

*n=6

Table 3: Precision data of levosimendan

Conc. (µg/ml)	Intra-day		Inter-day	
	Mean Conc. ± SD*	% RSD*	Mean Conc. ± SD*	% RSD*

3	2.9682±0.0168	0.5660	3.0152±0.0154	0.5107
4	3.9942±0.0236	0.5908	3.9694±0.0306	0.7709
5	5.0168±0.0156	0.311	4.9256±0.0287	0.5828

*n=6

Table 4: Stability data of levosimendan (bench top stored stock)

Time (hrs)	Conc. of drug (µg/ml)	Absorbance	% amount remaining	%RSD (n=6)
0	4	0.6156	100	0.8253
4		0.6098	99.06	0.4284
8		0.6052	98.31	0.9175
12		0.6022	97.82	0.5296
24		0.6004	97.53	0.6183
48		0.5914	96.06	0.7621

Table 5: Stability data of levosimendan (refrigerated stock)

Time (Days)	Conc. of drug (µg/ml)	Absorbance	% amount remaining	%RSD (n=6)
0	4	0.6156	100	0.9253
1		0.6084	98.83	0.5216
2		0.6021	97.81	0.8719
3		0.5965	96.90	0.9548
4		0.5906	95.94	0.3287
5		0.5832	94.74	0.8631

Table 6: Optical characteristics of levosimendan

Parameters	Values
$A^{1\%}_{1cm}$	153.82
Molar absorptivity (ε)	4311.26
Sandell's sensitivity (gm x 10^{-3}/cm^2x 0.001 absorbance unit)	0.0064

Table 7: Assay results of levosimendan injection

Brand name of Injection	Labeled amount of Drug (mg)	Mean Assay ±SD	% Label Claim	%RSD (n = 6)
Simdax®	12.5	12.3987±0.0628	99.19	0.5065

Fig. 8: Linearity graph of levosimendan

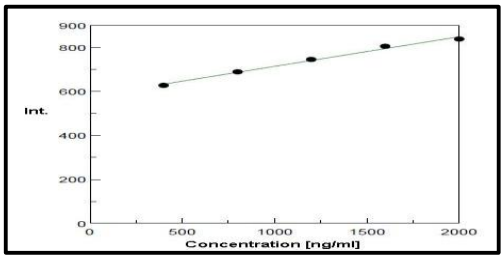

Table 9: Recovery data of levosimendan

Conc. of drug taken (ng/ml)	Conc. of standard added (ng/ml)	Mean Conc. found ± SD*	Mean Recovery (%)	% RSD*
800	400 (50%)	1192.54±14.4173	99.38	1.2089
800	800 (100%)	1578.31±8.7604	98.64	0.5551
800	1200 (150%)	1966.58±19.5295	98.32	0.9931

*n=6

Table 10: Precision data of levosimendan

Conc. (ng/ml)	Intra-day		Inter-day	
	Mean Conc. ± SD*	% RSD*	Mean Conc. ± SD*	% RSD*
800	794.56±8.9206	1.1227	789.92±5.3102	0.6723
1200	1203.81±11.8774	0.9867	1182.23±12.9176	1.0926
1600	1587.92±18.8627	1.1879	1591.59±14.2257	0.8938

*n=6

Table 11: Stability data of levosimendan (bench top)

Time (hrs)	Conc. of drug (ng/ml)	Intensity	% amount remaining	%RSD (n=6)
0	1200	751.62	100	0.8152
4		747.23	99.42	1.0475
8		735.76	97.89	0.4382
12		728.91	96.98	1.1729
24		720.27	95.82	0.9714
48		699.24	93.03	1.0236

Table 12: Stability data of levosimendan (refrigerated)

Time (Days)	Conc. of drug (ng/ml)	Intensity	% amount remaining	%RSD (n=6)
0	1200	751.62	100	0.8152
1		744.81	99.09	0.4084
2		737.24	98.08	1.0428
3		731.75	97.35	0.9815
4		727.52	96.79	1.2617
5		705.86	93.91	1.0629

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Fig. 9: Emission spectra of cerium (III) (formed after oxidation of levosimendan injection)

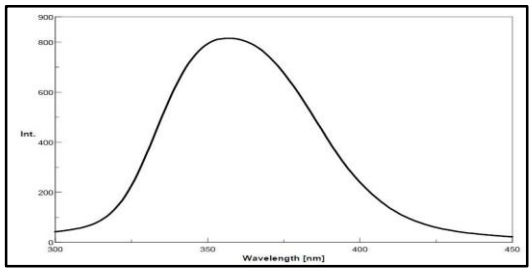

Table 13: Assay results of levosimendan injection by spectrofluorimetry

Brand name of tablets	Labeled amount of Drug (mg)	Mean Assay ±SD	% Label Claim	%RSD (n = 6)
Simdax®	12.5	12.3858 ±1.0527	99.08	0.8541

Table 14: Linearity data for levosimendan by HPTLC method

S. No	Concentration (ng/band)	Peak area
1	200	1108
2	400	2227
3	600	3144
4	800	3977
5	1000	4721

Fig. 10: Linearity graph of levosimendan

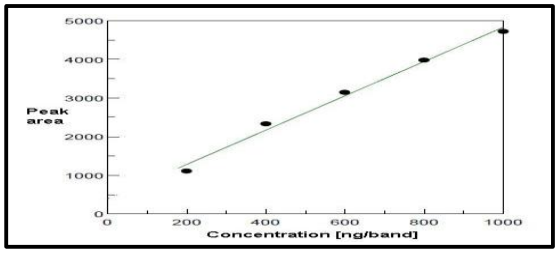

Table 15: Recovery data of levosimendan

Conc. of drug taken (ng/band)	Conc. of standard added (ng)	Mean Conc. ± SD*	Mean Recovery (%)	% RSD*
400	200 (50%)	598.9910±1.6108	99.83	0.2689
400	400 (100%)	800.7925±7.8131	100.09	0.9757
400	600 (150%)	999.7462±10.039	99.97	1.0042

*n=6

Table 16: Repeatability data of sample application of levosimendan

Concentration (ng/band)	Peak area	%RSD
600	3156	0.4772
	3153	
	3137	
	3132	
	3146	

	3174	

Table 17: Repeatability data of sample measurement of levosimendan

Concentration (ng/band)	Peak area	%RSD
600	3164	0.8587
	3172	
	3136	
	3183	
	3127	
	3197	

Table 18: Precision data of levosimendan

Conc. (ng/band)	Intra-day		Inter-day	
	Mean Conc. ± SD*	% RSD*	Mean Conc. ± SD*	% RSD*
400	398.9574±0.7758	0.1945	398.7553±1.3233	0.3318
600	599.2837±0.9170	0.1530	599.4133±1.2106	0.2019
800	800.2465±0.8909	0.1113	799.8736±1.0493	0.1312

*n=6

Table 19: Stability data of Levosimendan (bench top)

Time (hrs)	Conc. of drug (ng/band)	Peak area	% amount remaining	%RSD (n=6)
0	600	3145	100	0.3549
4		3073	97.71	0.2517
8		3046	96.85	0.1526
12		2987	94.98	0.2631
24		2845	90.46	0.5427
48		2675	85.06	0.4284

Table 20: Stability data of Levosimendan (Refrigerated)

Time (Days)	Conc. of drug (ng/band)	Peak area	% amount remaining	%RSD (n=6)
0	600	3145	100	0.2054
1		3097	98.47	0.1529
2		3054	97.11	0.3648
3		2958	94.05	0.4739
4		2926	93.04	0.1286
5		2879	91.54	0.3287

Table 21: Assay results of levosimendan injections by HPTLC method

Brand name of injections	Labelled amount of Drug (mg)	Mean Assay ±SD	% Label Claim	%RSD (n = 6)
Simenda®	12.5	12.4616±0.1086	99.69	0.8618

Table 22: Linearity data for levosimendan

S. No	Concentration (µg/ml)	Peak area
1	0.2	18768
2	0.4	37452
3	0.6	51559
4	0.8	68146
5	1	87338
6	1.2	108094

Table 23: Recovery data of levosimendan

Conc. of drug taken (µg/ml)	Conc. of standard added (µg/ml)	Mean Conc. ± SD*	Mean Recovery (%)	% RSD*
0.4	0.2 (50%)	0.5918±0.0084	98.63	1.4531
0.4	0.4 (100%)	0.7883±0.0121	98.54	1.5349
0.4	0.6 (150%)	0.9947±0.0194	99.47	0.9450

*n=6

Table 25: Precision data of levosimendan

Conc. (µg/ml)	Intra-day		Inter-day	
	Mean Conc. ± SD*	% RSD*	Mean Conc. ± SD*	% RSD*
0.6	0.5968 ±0.0076	1.2735	0.5994±0.0050	0.8352
0.8	0.7957 ±0.0084	1.0527	0.7960±0.0054	0.6790
1.0	0.9941 ±0.0104	1.0512	0.9984±0.0086	0.8650

*n=6

Table 26: Stability data of levosimendan (bench top)

Time (hrs)	Conc. of drug (µg/ml)	Peak Area	% amount remaining	%RSD (n=6)
0	1	87255	100	0.1026
4		86898	99.59	0.2108
8		86187	98.77	0.1325
12		85014	97.43	0.2652
24		82537	94.59	0.2347
48		78472	89.93	0.3264

Table 27: Stability data of levosimendan (refrigerated)

Time (Days)	Conc. of drug (µg/ml)	Peak Area	% amount remaining	%RSD (n=6)
0	1	87255	100	0.1026
1		86528	99.17	0.2329
2		85293	97.75	0.1413
3		83265	95.43	0.2362

4		82381	94.41	0.3138
5		81095	92.94	0.2104

Table 28: System suitability parameters of levosimendan

Retention time	Tailing factor	Plate count
11.1 min	1.253	2357

Table 29: Assay of levosimendan injections

Brand name of injections	Labeled amount of Drug (mg)	Mean Assay ±SD	% Label Claim	%RSD (n = 6)
Simenda®	12.5	12.4394±0.0878	99.52	0.7058

Table 30: Forced degradation results of levosimendan

Stress condition	% degradation		Retention time of Degradation product
	0 hrs	After 2 hrs	
Acid hydrolysis	18.02	37.29	---
Alkali hydrolysis	1.46	21.80	---
Neutral hydrolysis	6.46	18.74	Degradant peak at 4.8min & 8.6min
Oxidative degradation	14.11	100	Degradant peak at 4.8 min
Photodegradation	-	9.23	---

Fig. 28: Spectrum of levosimendan peak at 0 time of acid hydrolysis

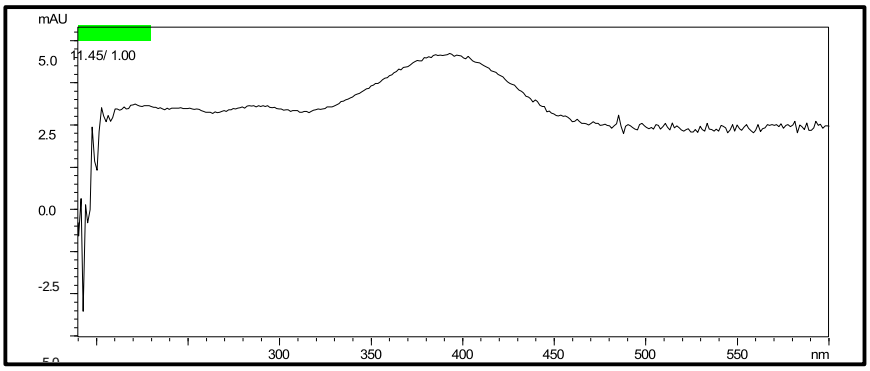

Fig. 32: Spectrum of levosimendan peak at 0 time of base hydrolysis

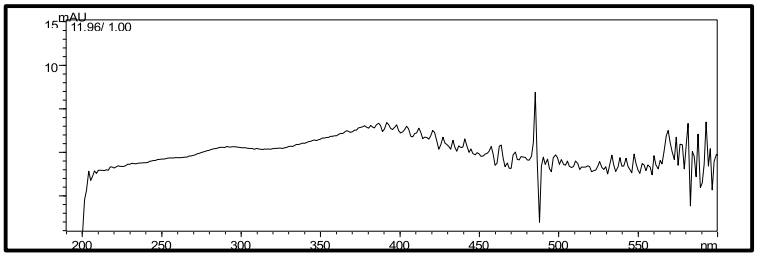

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Fig. 33: Chromatogram of levosimendan after 2 hrs of base hydrolysis

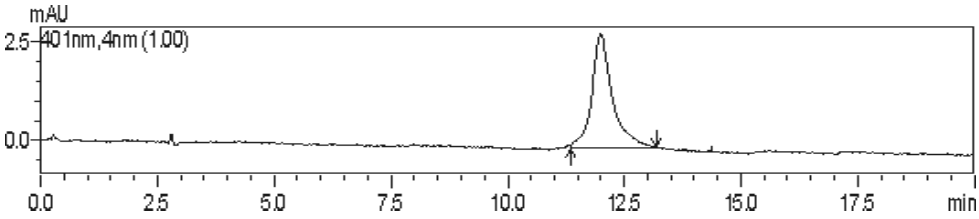


Fig. 34: Spectrum of levosimendan peak after 2 hrs of base hydrolysis

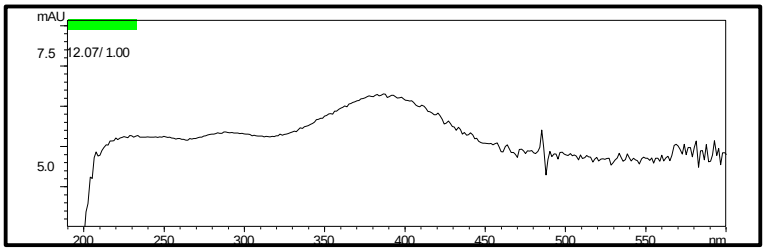


Fig. 36: Spectrum of levosimendan peak at 0 time of neutral hydrolysis

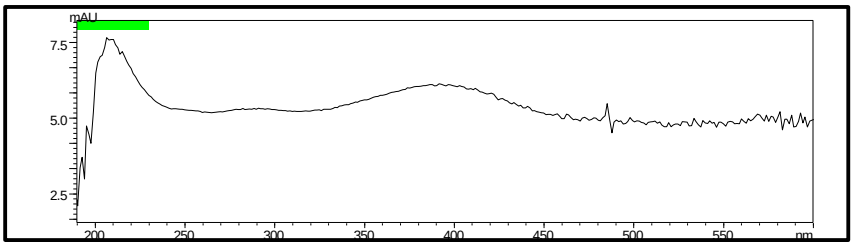
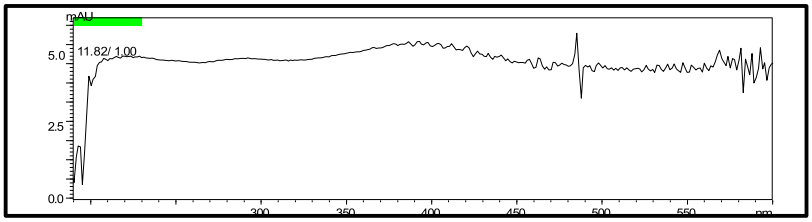


Fig. 38: Spectrum of levosimendan peak after 2 hrs of neutral hydrolysis



ig. 39: Neutral hydrolysis - spectrum of degradant peak at 4.8min

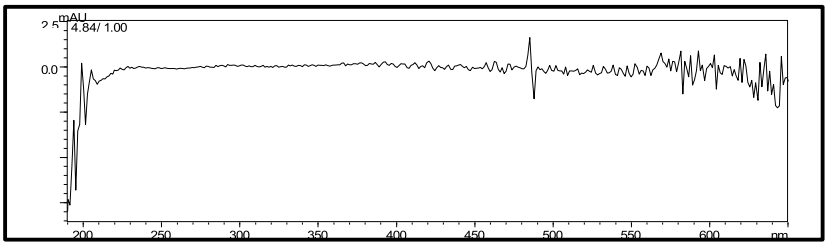


Fig. 40: Neutral hydrolysis - spectrum of degradant peak at 8.6min

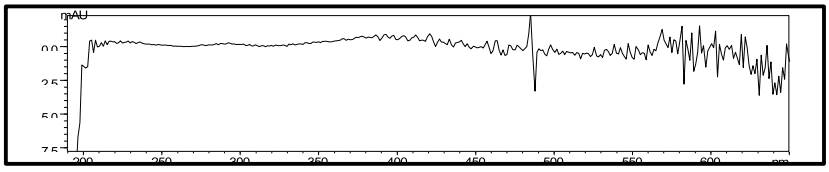


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Fig. 42: Spectrum of levosimendan peak at 0 time of oxidative degradation

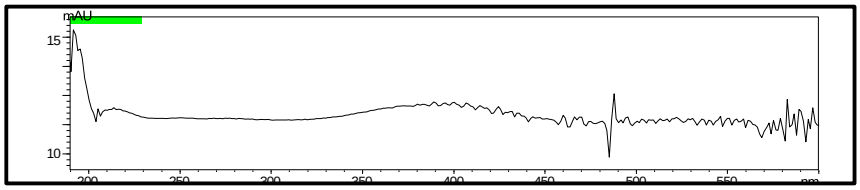

Fig. 44: Oxidative stress- spectrum of degradant peak at 4.8 min

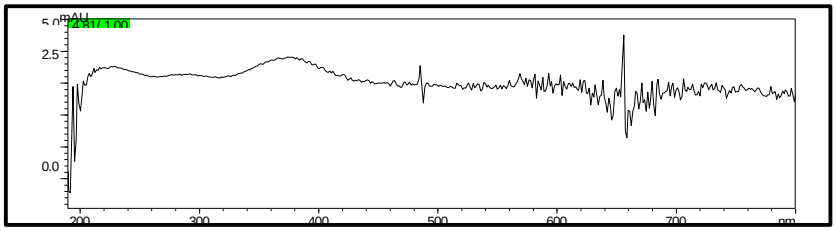

Fig. 46: Spectrum of levosimendan peak after 4 hrs Photolytic stress

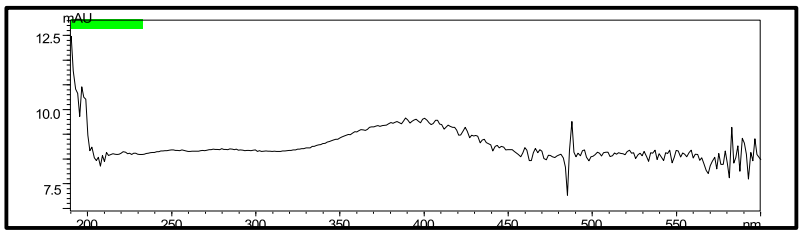

Fig. 47: Chromatogram of simultaneous elution of levosimendan with aspirin

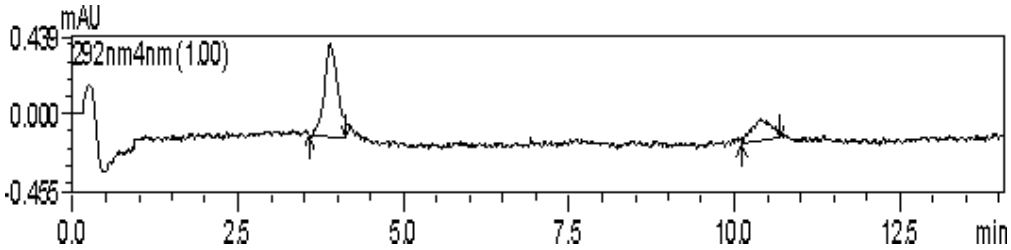

Fig. 48: Chromatogram of simultaneous elution of levosimendan with clopidogrel

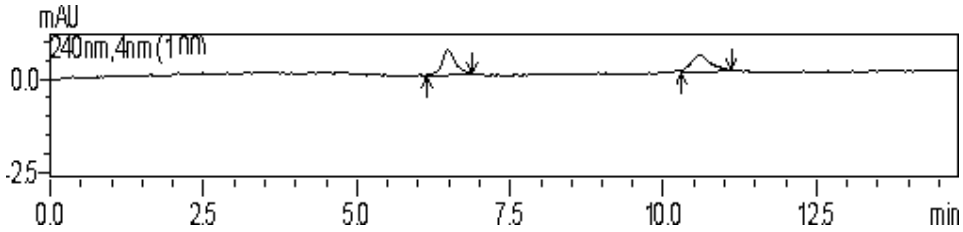

Fig. 49: Chromatogram of simultaneous elution of levosimendan with atorvastatin

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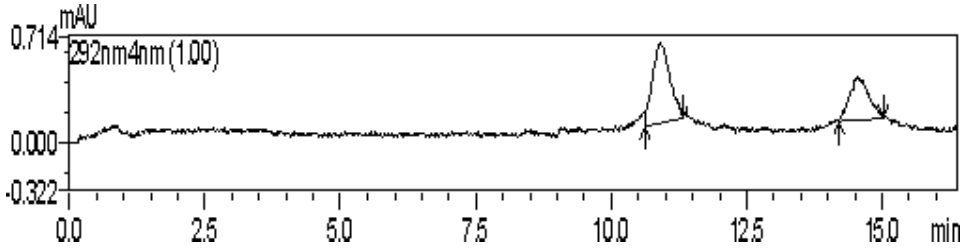


Fig. 50: Chromatogram of unbound levosimendan after equilibrium dialysis withprotein

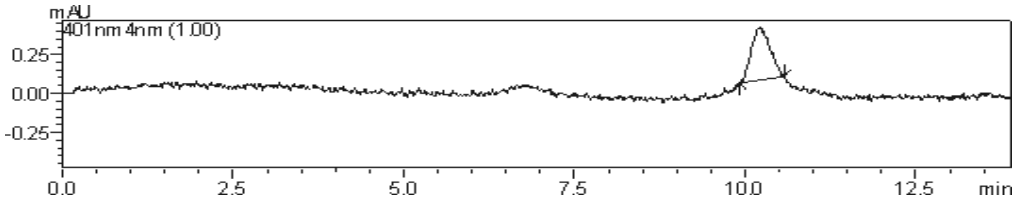
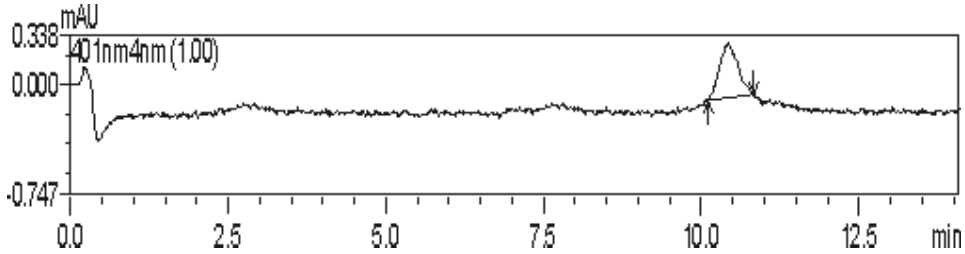


Fig. 51: Chromatogram of levosimendan unbound levosimendan after interaction with aspirin



Conclusion

A series of validated analytical methods, including UV spectrophotometry, spectrofluorimetry, HPTLC, and stability-indicating RP-HPLC, were successfully developed for the quantitative estimation of levosimendan in injection formulations. Each method demonstrated excellent linearity, accuracy, precision, and specificity, thereby confirming their suitability for routine quality control applications.

Among these, the RP-HPLC method showed the added advantage of being stability-indicating, capable of effectively resolving levosimendan from its degradation products under various stress conditions. Furthermore, its application in in-vitro drug interaction studies revealed significant displacement of levosimendan from protein binding sites by commonly co-administered drugs such as aspirin and atorvastatin, indicating a potential for pharmacokinetic interactions.

Overall, the developed methods are reliable, reproducible, and adaptable for routine laboratory analysis of levosimendan in both pure and formulation forms. The RP-HPLC method, in particular, is advantageous for advanced analytical applications, including interaction and stability studies.

5. ACKNOWLEDGEMENT

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