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Simultaneous separation and determination of coenzyme Q_{10} and its process related impurities by NARP-HPLC and atmospheric pressure chemical ionization-mass spectrometry (APCI-MS)

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Abstract

A non-aqueous reversed phase high performance liquid chromatographic (NARP-HPLC) method for determination of coenzyme Q_{10} in pharmaceutical preparations has been developed using Kromosil C_8 column with acetonitrile and isopropyl alcohol (84:16, v/v) as a mobile phase. Photodiode array (PDA) detector set at 210 nm was used for monitoring of the eluents. The method is simple, rapid, selective and capable of separating all process impurities at trace level with detection limits <0.1 μg/ml. It has been validated with respect to accuracy, precision, linearity, and limits of detection and quantification. The linearity range was 50–300 μg/ml. The percentage recoveries ranged from 95.10 to 101.02. The method was found to be suitable not only for monitoring the reactions during the process development but also quality assurance of coenzyme Q_{10}. For identification of related substances atmospheric pressure chemical ionisation-mass spectrometry (APCI-MS) was used.

Keywords: Coenzyme Q_{10}; Dietary supplement; Related substances; Non-aqueous reversed phase HPLC; APCI-MS

1. Introduction

Coenzyme Q_{10} (CoQ_{10}) is an essential vitamin-like nutrient for cell respiration and electron transfer to control the production of energy in the cells of heart [1,2]. It acts as a powerful antioxidant and membrane stabilizer in preventing cellular damage resulting from normal metabolic processes. It is naturally synthesized and occurs in all cells in the human body, but its rate of production falls with age. It is found in food, especially meat, but in very small amounts as thermal processing destroys it [3]. The use of CoQ_{10} as a dietary, nutraceutical supplement has increased dramatically in the last decade [4,5]. It has potential preventive and therapeutic effects in many diseases like cancer [6,7], cardiovascular [6,8,9] and neurodegenerative disorders [10], acquired immunodeficiency syndrome (AIDS) [6] and Parkinson's disease [11–13]. It is also known to be an energy booster and immune system enhancer [6,14]. Recently, the commercial formulations containing coenzyme Q_{10} have gained increasing popularity in health management [15,16].

A through literature search has revealed that only a few analytical methods are available for determination of CoQ_{10} in bulk drugs and pharmaceuticals. Derivative UV spectrophotometry [17] FT-IR [18] and HPLC were used for analysis of CoQ_{10} in pharmaceuticals and human plasma [19–21]. However, none of these methods address to the problem of separation and determination of process related impurities, which are most likely to be present in the finished products of CoQ_{10}. Further to the best of our knowledge no method for determination of its impurities has been reported either in bulk drugs or pharmaceuticals. Thus there is a great need for analytical methods, which will be helpful to monitor the levels of impurities in the finished products of CoQ_{10} during process development. In the present study, the separation and determination of its process related impurities was examined by non-aqueous reverse-phase high performance liquid chromatography (NARP-HPLC) using a C_8 column connected to a photodiode array (PDA) detector set at 210 nm. The related substances were identified by APCI-MS.

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2. Experimental

2.1. Materials and reagents

All the reagents were analytical reagent grade unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA), HPLC-grade acetonitrile, isopropyl alcohol (S.D. Fine Chem., Mumbai, India) were used. Samples of 2,3-dimethoxy-5-methyl-*p*-benzoquinone from Sigma, USA, coenzyme Q_{10} extra pure 99% from Sisco Research Laboratories, Mumbai, India were purchased. Solanesol [22], solanesyl acetone, isodecaprenol were synthesized in our laboratory.

2.2. Apparatus

The HPLC system 1 composed of two LC-10AT VP pumps, an SPD-10Avp diode array detector a SIL-10AD VP auto injector, a DGU-12A degasser and SCL-10 A VP system controller (all from Shimadzu, Kyota, Japan). A reverse-phase (KR100-5C8) Kromasil C_8 (Eka Chemicals, Bohus, Sweden) column (250 × 4.6 mm × 5 μm) was used for separation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packed, Waldron, Germany) computer system.

The HPLC system 2 consisting of two LC-20AT pumps, an SPD-M20A diode array detector, a SIL-20AC auto sampler, a DGU-20A_3 degasser and CBM-20A communications bus module (all from Shimadzu, Kyoto, Japan) was used. A reversed phase Kromasil C_8 (Eka Chemicals, Bohus, Sweden) column (250 × 4.6 mm × 5 μm) was used for separation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan).

2.3. Chromatographic conditions

The mobile phase was acetonitrile and isopropyl alcohol (84:16, v/v). Before delivering in to the system it was filtered through 0.45 μm, PTFE filter and degassed using vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0 ml/min at 50 °C. The chromatograms were recorded at 210 nm using an SPD-M10Avp diode array detector.

2.4. APCI-MS

The APCI-MS experiments were performed using a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA), equipped with APCI and ESI sources. The data acquisition was carried under the control of Xcalibur software in positive ion mode. Conditions for the APCI-MS analyses were as follows: vaporization temperature: 300 °C; sheath gas (N_2): 65 ml/min;

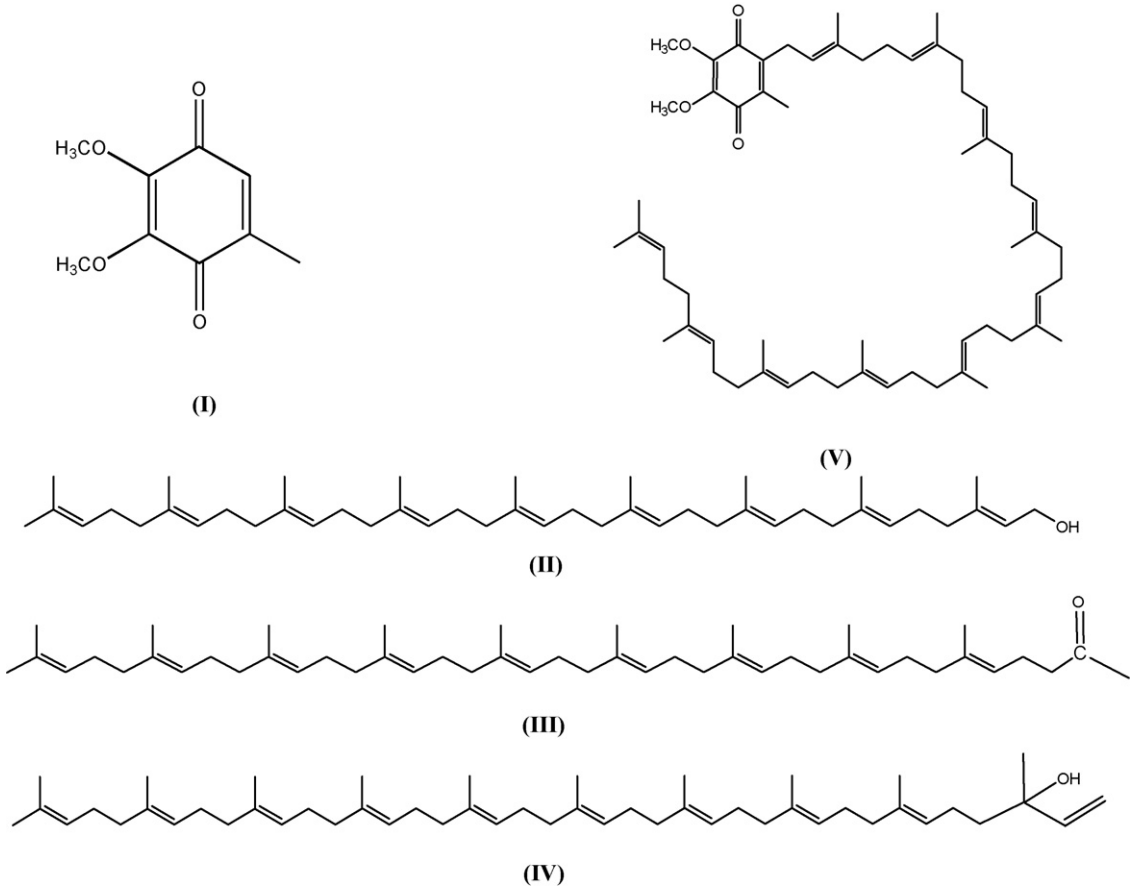

Fig. 1. Chemical structures of CoQ_{10} (V) and its related substances (I) 2,3-dimethoxy-5-methyl-*p*-benzoquinone, (II) solanesol, (III) solanesyl acetone and (IV) isodecaprenol.

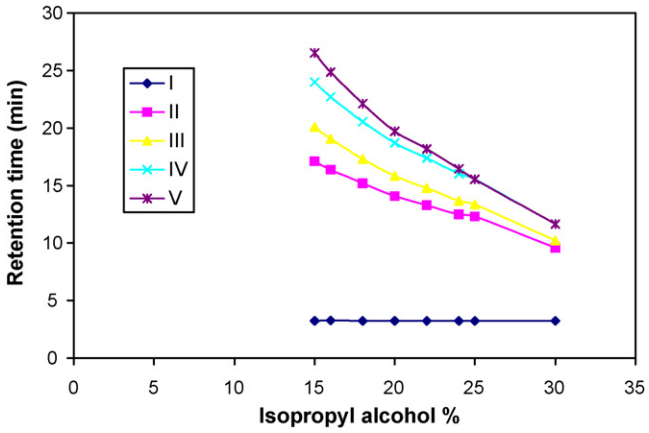

Fig. 2. Effect of organic modifier on retention of CoQ_{10} and its related substances.

aux gas (N_2): 20 ml/min; capillary temperature: 150 °C; discharge current: 5.00 μA; discharge voltage: 2.52 kV; capillary voltage: 15 to 20 kV; scan range: 50–2000 *m/z*.

2.5. Analytical procedures

Solutions (1 mg/ml) of coenzyme Q_{10} and the impurities were prepared by dissolving known amounts in methanol. The solutions were adequately diluted with mobile phase to study accuracy, precision, linearity, limits of detection (LOD) and quantitation (LOQ).

3. Results and discussion

3.1. Method development

The chemical structures of CoQ_{10} (V) and its process related impurities (I, II, III and IV) are shown in Fig. 1. The present study was aimed at developing of a chromatographic system capable of eluting and resolving CoQ_{10} and its potential impurities originating from the synthesis. Solanesol, solanesyl acetone, isodecaprenol were structurally similar and hydrophobic in nature. Generally the retention of hydrophobic compounds on chemically bonded reverse phases is large and non-aqueous solvents such as methanol, acetonitrile and THF are used to accomplish the elution in an acceptable time. For example the separation of fats, carotinoids and sterols are usually carried out by non-aqueous reversed phase chromatography [23,24]. Under such conditions the homologues are better resolved than on silica, which is another advantage of reversed phase separations. Thus non-aqueous reversed phase HPLC was carried out to separate process intermediates effectively from coenzyme Q_{10}. Initially, ACN with THF, MeOH, EtOH were tried. When methanol instead of ACN was tried baseline noise with tailing was observed. Different

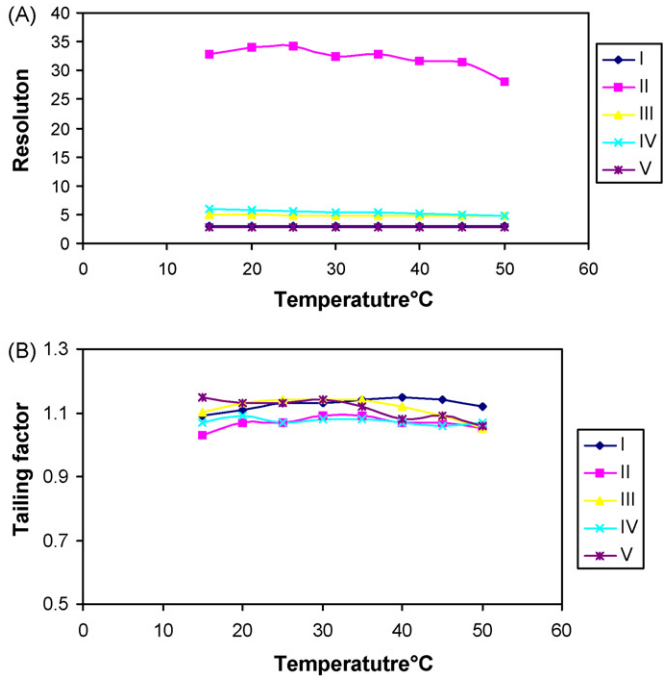

Fig. 3. Effect of temperature on (A) resolution and (B) tailing of peaks.

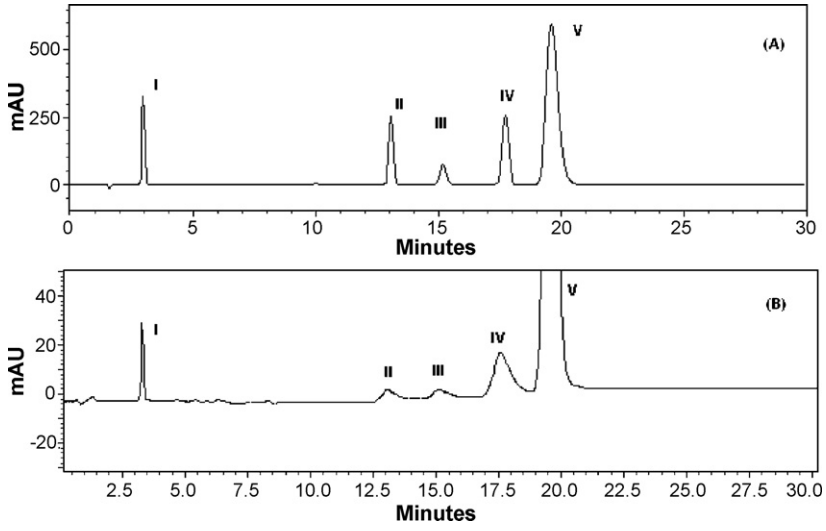

Fig. 4. Typical chromatograms of coenzyme Q_{10} (V) (A) spiked with related substances and (B) one of the commercial formulations.

Table 1
System suitability data

Compound	k'	As	RRT	Rs	N
I	0.97	1.06	0.156	2.95	15,168
II	7.72	1.05	0.673	28.04	15,255
III	9.14	1.05	0.783	4.78	16,780
IV	10.83	1.07	0.913	4.87	15,341
V	11.96	1.06	1.000	2.82	15,701

k', capacity factor; As, tailing factor; RRT, relative retention time; Rs, resolution; N, number of theoretical plates.

compositions of IPA and ACN were tried for better separation.

3.2. Optimization of chromatographic conditions

3.2.1. Column selectivity

Initially different C_{18} columns were tried. Good separation was achieved with ACN: IPA (70:30, v/v) with in 30 min. Later, C_8 column was used to separate all the impurities and CoQ_{10} with in 20 min using ACN:IPA (80:20, v/v). It was observed that C_8 (Kromasil C_8) was suitable to well separate with less tailing. So it was chosen for further development. All the impurities of CoQ_{10} were subjected to separation by NARP-HPLC on a Kromasil C_8 column with ACN-IPA as an eluent.

3.2.2. Effect of organic modifier

The separation of impurities II, III and IV became critical as they eluted very close to each other. When IPA was used as an organic modifier resolution was improved for compounds II, III, IV from compound CoQ_{10}. IPA was tried from 10 to 50%. It was found that 20% IPA was suitable for good separation. Further, optimization was carried out by changing IPA percentage 15–20%. It was found that 16% IPA was more suitable for separation of impurities from CoQ_{10}. The optimized percentage of IPA is shown in Fig. 2.

3.2.3. Effect of temperature

The column was maintained at different temperatures ranging from 15 to 50 °C in a thermostated oven. Retentions were decreased slightly with increasing temperature, and peaks became very sharp and good resolution was observed (Fig. 3A). Tailing was reduced with increasing temperature for all the compounds (Fig. 3B), and it was minimum at 50 °C and run time was reduced to 20 min.

Table 3
Intermediate precision: assay variation of coenzyme Q_{10}

Intra-day			
Day 0			
Mean of concentration (mg/ml: $n = 3$)	0.0516	0.1522	0.2498
S.D.	0.0002	0.0002	0.0003
R.S.D. (%)	0.38	0.10	0.12
Day 1			
Mean of concentration (mg/ml: $n = 3$)	0.0500	0.1500	0.2490
S.D.	0.0007	0.0007	0.0004
R.S.D. (%)	1.32	0.48	0.16
Day 2			
Mean of concentration (mg/ml: $n = 3$)	0.0503	0.1471	0.2497
S.D.	0.0002	0.0004	0.0001
R.S.D. (%)	0.41	0.27	0.04
Inter-day			
Mean of concentration (mg/ml: $n = 3$)	0.0496	0.1474	0.2495
S.D.	0.0002	0.0003	0.0003
R.S.D. (%)	0.30	0.17	0.12
Analyst 1			
Mean of concentration (mg/ml: $n = 3$)	0.0514	0.0151	0.2494
S.D.	0.0004	0.0008	0.0012
R.S.D. (%)	0.84	0.52	0.46
Analyst 2			
Mean of concentration (mg/ml: $n = 3$)	0.0495	0.0149	0.2492
S.D.	0.0003	0.0012	0.0007
R.S.D. (%)	0.61	0.83	0.28
HPLC system 1			
Mean of concentration (mg/ml: $n = 3$)	0.0508	0.1503	0.2506
S.D.	0.0009	0.0008	0.0092
R.S.D. (%)	1.82	0.55	0.37
HPLC system 2			
Mean of concentration (mg/ml: $n = 3$)	0.0496	0.1516	0.2490
S.D.	0.0002	0.0006	0.0032
R.S.D. (%)	0.41	0.39	1.26

Finally, separation was carried out on the Kromasil C_8 column maintained at 50 °C with an isocratic elution using ACN:IPA as a mobile phase and PDA detector set at 210 nm. A typical chromatogram showing the separation of 10% (w/w) of each of related impurities spiked to CoQ_{10} at the specified relative concentrations of 300 μg/ml is shown in Fig. 4. It could be seen from Fig. 4 that all the compounds were eluted and separated with good peak shapes and resolutions. The developed method was validated with respect to accuracy, precision and linearity as per ICH guidelines [25].

Table 2
Recovery data

Sample	Recovery[a] ± R.S.D. (%)					
Amount added (μg/ml)	0.3	0.5	0.7	1.0	1.5	2.0
I	98.20 ± 0.55	98.86 ± 0.84	99.19 ± 0.72	97.00 ± 1.03	99.55 ± 1.02	95.10 ± 0.47
II	98.65 ± 0.58	98.60 ± 0.40	99.28 ± 0.57	98.00 ± 1.02	99.33 ± 0.67	98.66 ± 0.77
III	101.02 ± 0.62	98.86 ± 0.42	99.19 ± 0.29	98.00 ± 1.02	98.66 ± 1.35	98.50 ± 1.01
IV	99.54 ± 0.36	99.26 ± 0.30	99.23 ± 0.50	97.33 ± 1.18	98.44 ± 1.03	99.00 ± 0.50
Amount added (μg/ml)	50	75	100	150	225	300
V	99.2 ± 0.40	99.42 ± 0.47	100.00 ± 0.05	99.93 ± 0.20	99.71 ± 0.09	99.63 ± 0.29

[a] Average of three determinations.

Table 4
Precision data

Sample	Precision type	Concentration 0.3 μg/ml	R.S.D. (%)	Concentration 0.5 μg/ml	R.S.D. (%)	Concentration 0.7 μg/ml	R.S.D. (%)	Concentration 1.0 μg/ml	R.S.D. (%)	Concentration 1.5 μg/ml	R.S.D. (%)	Concentration 2.0 μg/ml	R.S.D. (%)
Intra-day ($n = 3$)													
I		0.31	0.50	0.51	0.29	0.71	0.21	1.11	1.36	1.51	1.00	2.12	0.98
II		0.30	1.50	0.51	1.32	0.71	0.29	1.12	0.51	1.51	0.66	2.11	0.72
III		0.32	1.71	0.51	0.40	0.72	0.50	1.13	1.34	1.51	0.20	2.15	1.41
IV		0.31	1.11	0.52	0.22	0.71	0.21	1.16	0.98	1.52	0.13	2.20	0.45
Inter-day ($n = 3$)													
I		0.31	0.80	0.52	1.36	0.71	0.21	1.12	1.78	1.51	0.38	2.08	1.27
II		0.32	0.31	0.52	0.79	0.72	0.27	1.19	1.27	1.51	0.40	2.22	1.19
III		0.30	1.89	0.50	1.73	0.73	0.20	1.27	1.97	1.52	1.06	2.13	1.95
IV		0.32	1.40	0.52	0.19	0.71	0.21	1.16	0.86	1.51	0.66	2.12	1.18
Inter-analyst ($n = 3$)													
I	Analyst 1	0.31	0.49	0.51	0.88	0.71	0.21	1.03	1.47	1.51	0.23	2.15	1.23
	Analyst 2	0.29	1.17	0.51	0.58	0.71	0.35	1.02	0.98	1.51	0.31	2.09	0.72
II	Analyst 1	0.31	0.48	0.50	1.76	0.71	0.21	1.13	1.34	1.51	0.33	2.14	0.71
	Analyst 2	0.31	1.38	0.51	0.19	0.72	1.04	1.16	1.31	1.52	0.30	2.19	1.83
III	Analyst 1	0.32	0.47	0.52	0.48	0.72	0.55	1.17	1.29	1.50	1.01	2.08	1.44
	Analyst 2	0.31	0.48	0.51	0.99	0.71	0.21	1.24	2.10	1.53	1.72	2.12	0.97
IV	Analyst 1	0.31	0.65	0.51	0.29	0.71	0.49	1.16	1.31	1.51	0.13	2.17	1.06
	Analyst 2	0.31	1.11	0.52	0.61	0.71	0.14	1.22	0.81	1.51	0.38	2.14	0.71
Inter-instrument ($n = 3$)													
I	HPLC system 1	0.31	1.00	0.50	1.68	0.70	1.67	1.00	1.28	1.51	0.20	2.17	0.79
	HPLC system 2	0.31	1.11	0.51	0.29	0.71	0.29	1.16	2.58	1.49	1.39	2.14	0.97
II	HPLC system 1	0.31	0.48	0.51	0.33	0.70	1.07	1.21	0.34	1.51	0.15	2.08	1.68
	HPLC system 2	0.31	0.65	0.50	1.78	0.71	0.41	1.13	1.34	1.48	1.69	2.13	0.71
III	HPLC system 1	0.31	0.54	0.51	0.68	0.71	0.29	1.12	0.51	1.51	0.13	2.14	0.93
	HPLC system 2	0.31	1.18	0.51	1.07	0.72	0.34	1.16	1.78	1.50	1.76	2.11	1.44
IV	HPLC system 1	0.30	1.42	0.50	0.50	0.71	0.21	1.12	1.35	1.51	0.20	2.15	1.61
	HPLC system 2	0.31	0.65	0.51	0.29	0.70	0.70	1.13	1.34	1.50	1.01	2.12	0.71

Table 5
Linearity data

Compound	Range (μg/ml)	Regression equation	r^2	LOD	LOQ
I	0.05–2.0	$y = 33477x - 976.36$	0.9981	0.013	0.043
II	0.10–2.0	$y = 53003x + 813.71$	0.9987	0.024	0.082
III	0.25–2.0	$y = 24458x + 4545.3$	0.9988	0.068	0.224
IV	0.10–2.0	$y = 122709x - 4548.1$	0.9992	0.023	0.075
V	50–300	$y = 56966x + 637831$	0.9999	0.090	0.297

3.3. Validation

3.3.1. System suitability

The system suitability was conducted by using 0.1% of all impurities spiked to CoQ_{10} and evaluated by making five replicate injections. The system was deemed to be suitable for use if the tailing factor for CoQ_{10} and its impurities were ≤1.2, the resolution was >1.5 and column plate numbers for main peak were >15,000. Synthetic mixtures and process samples were analyzed under identical conditions. The quantities of impurities and assay of CoQ_{10} were calculated from their respective peak areas (Table 1).

3.3.2. Accuracy

The recoveries of I, II, III and IV were determined by spiking each impurity at six different levels ranging from 0.3 to 2.0 μg/ml to CoQ_{10} (V) at the concentration of 300 μg/ml. The recovery range and R.S.D. for all impurities were found to be 95–100% and <5%, respectively (Table 2). Similarly the accuracy in determination of the assay of CoQ_{10} was checked at six concentration levels i.e. 50, 75, 100, 150, 225 and 300 μg/ml each in triplicate for 3 days and the percentage recoveries are recorded in Table 2. The R.S.D. were <5%.

3.3.3. Precision

The precision of the method was tested by six ($n = 6$) injections of CoQ_{10} spiked with 0.1% (w/w) of each impurity. The R.S.D. in determination of retention time (t_R) and, peak area R.S.D. ranged from 0.16 to 1.10 %. The precision in determination of assay was studied by repeatability, intermediate precision. Repeatability is the intra-day variation in assay obtained at different concentration levels of CoQ_{10} and expressed in terms of R.S.D. calculated for each day. The R.S.D. values were found to be below 1.5%, indicating a good repeatability (Table 3). The intermediate precision is the inter-day variation at the same concentration levels determined at successive days. The inter-day variations calculated for each concentration level from the data of 3 days were expressed in terms of R.S.D. values. The data for analyst-to-analyst, instrument-to-instrument variation of Q_{10} assay was summarized in Table 3. The intermediate precision for the impurities were calculated at six concentration levels (0.3, 0.5, 0.7, 1.0, 1.5 and 2 μg/ml) for 3 days. The data for intra- and inter-day, analyst (Analyst 1 and Analyst 2), instrument (HPLC system 1 and HPLC system 2) precisions of the impurities were summarized in Table 4. The data obtained was within 2% R.S.D.

3.3.4. Linearity

The linearity of detector response to different concentrations of impurities were studied by analyzing CoQ_{10} spiked at eight levels ranging from 0.05–0.1 to 2 μg/ml. Similarly, the linearity of CoQ_{10} was also studied by preparing standard solutions at eight different levels ranging from 50 to 300 μg/ml. The data were subjected to statistical analysis using a linear-regression model. The standard deviation of slope and intercept were calculated and shown in Table 5. The results have indicated a good linearity.

3.3.5. Limits of detection and quantitation

Limits of detection and quantitation represent the concentration of the analyte that would yield signal-to-noise ratio of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The results are given in Table 5. The quantitation limit was subsequently validated by the analysis of a suitable number of samples near at quantitation limit. The results are given in Table 6.

Table 6
Accuracy and precision determination at LOQ values

Compound	Sample concentration				Average recovery[a]	% Recovery	%R.S.D.
	Taken (μg/ml)	Found value[b] ($n = 3$)					
I	0.05	0.049	0.049	0.050	0.049	98.66	1.17
II	0.10	0.100	0.099	0.098	0.099	99.00	1.01
III	0.25	0.250	0.245	0.249	0.248	99.20	1.06
IV	0.10	0.980	0.970	0.990	0.980	98.00	1.02
V	0.30	0.298	0.299	0.297	0.298	99.33	0.33

[a] Average recovery from three samples.
[b] Average of three determinations (n = three number of samples).

Table 7
Results of analysis of bulk drugs/formulations by HPLC

S. No	Sample	Impurities (%)				Assay (w/w)
		I	II	III	IV	
1	Bulk drug	0.12	0.2	0.21	0.3	99.10
2	Formulation	0.2	0.1	0.3	0.36	99.03

3.4. Assay of coenzyme Q10 in capsule formulations

Five capsules were quantitatively transferred in to a separating funnel 50 ml of deionized distilled water and 50 ml of *n*-hexane were added. The mixture was shaken vigorously and organic layer was transferred to a 250-ml volumetric flask. The extraction was repeated twice. The hexane extract was adjusted up to 250 ml with isopropyl alcohol. The working solutions were prepared by the appropriate dilutions with isopropyl alcohol. For determining the impurities, the same solution was used. The results are recorded in Table 7. A typical chromatogram is shown in Fig. 4B. The peaks were identified by injecting and comparing with the retention times of the individual compounds and APCI-MS spectra of related substances. The concentrations of impurities relative to CoQ_{10} were in the range 0.1–1.0% (Table 7). The assay for determining the CoQ_{10} was carried out by diluting the above solutions to 25–100 μg/ml with the mobile phase. Different batches of CoQ_{10} (V) were analyzed and results are recorded in Table 8.

The impurities with more than 0.1% area at retention times 3.01, 13.07, 15.21, 17.75, 19.43 min were detected. In order to identify these impurities APCI-MS was used. The MS analysis carried out in positive ion mode using atmospheric pressure chemical ionization technique. Out of which, one impurity at 3.01 had perfectly matched with the retention time and fragmentation pattern of (I) with protanated molecular ion *m/z* 183 (100%) and daughter ions *m/z* 165 and 137. Another impurity at 13.07 perfectly matched with fragmentation pattern of (II), which showed *m/z* 613 ($M–H_2O$) with daughter ions at 577, 219 was identified as (II). Impurity at 15.21 min, matches with retention time and shows its molecular ion at *m/z* 671, it conforms the impurity as III. Another peak at 17.75 shows *m/z* at 681($M–H_2O$), this supports the impurity as IV. In positive mode CoQ_{10} had shown as a molecular ion at *m/z* at 863. Its daughter ions found at 663, 391, 253. The APCI-MS spectra of CoQ_{10} and its potential impurities are shown in Fig. 5.

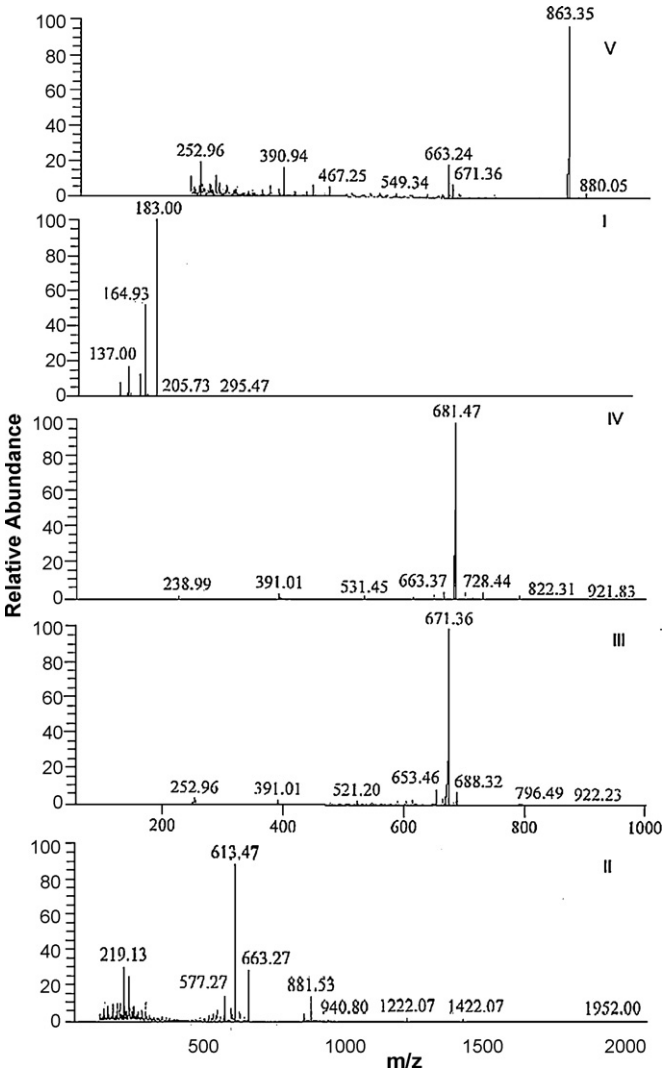

Fig. 5. APCI-MS spectra of (I) 2,3-dimethoxy-5-methyl-*p*-benzoquinone, (II) solanesol, (III) solanesyl acetone, (IV) isodecaprenol and (V) coenzyme Q_{10}.

Table 8
Assay of coenzyme Q10 in soft gelatin capsules

S. No.	Injection	Concentration of coenzyme Q_{10} (mg/ml)			R.S.D (%)
		Taken	Recovered	% Recovery	
I	1	0.0989	0.0986	–	–
	2	0.0989	0.0983	99.66	0.25
	3	0.0989	0.0988	–	–
II	1	0.0495	0.0510	–	
	2	0.0495	0.0499	100.33	1.46
	3	0.0495	0.0496	–	–
III	1	0.0246	0.0244	–	–
	2	0.0246	0.0243	97.6	0.41
	3	0.0246	0.0245	–	–

4. Conclusions

An isocratic NARP-LC method has been developed and validated for evaluation of purity of coenzyme Q_{10} in bulk drugs and formulations. The developed method is selective, sensitive, accurate and precise. The method is also capable of detecting process related impurities, which may be present at trace level in the finished products. The impurities were identified by APCI-MS in the bulk drugs and formulations.

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