Determination of DL Methionine in Soybean Natural Extract and Pharmaceutical Preparation by New HPLC Method and Detection of its Antioxidant Activity

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Abstract: The superoxide scavenging activity of DL methionine has been studied in vitro using PMS-NADH (phenazine methosulfate- NADH) system, to test the direct superoxide scavenging effect of DL methionine itself without converting it to any antioxidant molecule. Another method for detecting the antioxidant activity of DL methionine is iron thiocyanate method, it has been applied to detect whether DL methionine itself can prevent oxidation of Fe^{2+} to Fe^{3+} ions induced by peroxides, also a new HPLC–UV method was developed for the analysis of DL methionine in pure form, in multi ingredient dosage form with paracetamol and in Soybean extract . An excellent linearity ($r^2$0.9986), good intra-day precision (0.296) and inter-day reproducibility (0.485) were obtained. [Journal of American Science 2010;6(9):331-339]. (ISSN: 1545-1003).

Keywords: superoxide; methionine; iron thiocyanate; reproducibility

1. Introduction:

Methionine is an essential amino acid with an important role in biological methylation reactions. It constitutes the main supply of sulphur in the diet, preventing disorders in hair, skin or nails. Moreover, it helps to reduce cholesterol levels by increasing the lecithin production in liver. It acts as a natural chelating agent for heavy metal[i], it also acts as hepatoprotectant, antidote in acetaminophen poisoning and urinary acidifier[ii]. DL methionine is known as an antioxidant, as it acts as a precursor amino acid for important antioxidant molecules such as glutathione, cysteine and taurine which protect the cells from oxidative damage and play vital role in detoxification. In addition, methionine has been shown to chelate lead and remove it from tissues, it also has the hydroxyl and peroxynitrite radicals scavenging ability.

Homocysteine (Hcy), a thiol formed by demethylation of DL methionine, is at moderately high levels, a known independent risk factor for atherosclerosis and increased vascular dysfunction. However, Hcy, contains a thiol group, displays an antioxidant effect on cellular systems at micromolar levels.

For the determination of DL methionine, analysis is performed by voltammetric methods [iii,iv], Potentiometry[v], spectrophotometry[vi,vii], GC[viii], Capillary Electrophoresis[ix] and HPLC methods[x,xi,xii,xiii]

In a research program dealing with the development of new HPLC method for the analysis of DL methionine, we developed and validated a simple and accurate method for the analysis of DL methionine using different mobile phase of methanol: 0.05M phosphate buffer at PH 3.2 , this method was applied in pure form, in multiingradient formula with paracetamol and in Soybean extract. The antioxidant power of DL methionine has been evaluated using different in vivo and invitro techniques[xiv,xv,xvi]. In our work the superoxide scavenging effect of DL methionine alone and in presence of paracetamol has been studied, further, iron thiocyanate method for detecting its antioxidant activity has been applied.

2. Materials and Methods:

2.1. Instrumentation

- HPLC (BIO-TEK) Kontron instrument equipped with a model series 422 pump, knauer injector with a 50 µl loop and a 540+ photodiode array detector. Data acquisition was performed on a model kroma system 2000
- Absorption spectra were recorded on Double beam Shimadzu (Japan) 1601.
  Pc UV-VIS spectrophotometer connected to a computer fitted with UVPC personal spectroscopy software version 3.7, using matched quartz cuvettes in a thermostated cell holder. Measurements took place at 25 C (±0.2)
- Column cc 250/4.6 nucleosil 120-5 C_{18}
- 0.45µm Disposable membrane filters
- Jenway pH meter 3310 pH/ mV/ C meter.

2.2. Materials

DL methionine pure sample was kindly supplied by HIKMA pharmaceutical Co., Giza, Egypt. Impurities were reported to be less than 0.2%, which contain (Cl^-, SO_4^{--}) less than 200ppm and heavy metals less than 20 ppm, Hepamol® tablet produced by HIKMA pharmaceutical Co., Giza, Egypt, Batch no. 101, labeled to contain 100 mg DL methionine was obtained from the local market. Methanol (Sigma Gmbh, Germany). Potassium phosphate dibasic anhydrous (Ridel-deHaën, Sigma-Aldrich, Germany). Water (Honil limited). Orthophosphoric acid (Ridel-deHaën, Sigma-Aldrich, Germany). Linoleic acid (Sigma), 4×10^{-2} M in ethanol.

Ethanol (Merck). Concentrated HCl (Merck). Ammonium thiocyanate (Prolabo),30% W/V in ethanol, Ferrous chloride (Prolabo). 0.1% w/v in 3.5% HCl.

Ferrous Sulfate (sigma)(0.1% w/v in 3.5% HCl). Phenazine methosulphate (PMS) (Sigma, Germany)(60 µM in phosphate buffer). Nitro Blue Tetrazolium (NBT) (Sigma, Germany) (150 µM in phosphate buffer). Nicotinamide adenine dinucleotide (NADH) (Sigma, Germany)(486 µM in phosphate buffer). Disodium hydrogen phosphate (Adwiac).

2.3. Procedures

2.3.1. HPLC Technique

Chromatographic separation was carried out using Column nucleosil C_{18}under isocratic condition with a mixture of methanol: 0.05M phosphate buffer at PH 3.2 (30:70 v/v) at a flow rate of 1.0 $ml.min^{-1}$ The operative wavelength was set at 220 nm

2.3.1.1. Validation procedure

2.3.1.1.1. Linearity

Accurately measured volumes (1-4 ml) of stock standard solution were transferred into a series of 10 ml volumetric flasks, diluted to volume with water to obtain a concentration range of 100-400 $\mu g.ml^{-1}$. A 50 µl volume of each solution was injected, in triplicates; separated using the chromatographic conditions described above and average peak areas were calculated. The calibration curve representing the relationship between average peak area and corresponding concentration was plotted as shown in (figure 1) and the regression equation was computed.

2.3.1.1.2. Accuracy

The previously mentioned procedure under linearity was repeated for the analysis of different concentrations of pure DL methionine .The concentrations were calculated using the corresponding regression equation and the percentage recoveries were then calculated.

2.3.1.1.3. Precision

2.3.1.1.3.1. Repeatability

The intraday variation was evaluated by applying the previously mentioned procedure under linearity for the analysis of 100,120,200 $\mu g.ml^{-1}$ DL methionine solution (n=6). The concentrations were calculated using the corresponding regression equation and the percentage recoveries and standard deviations were then calculated.

2.3.1.1.3.2. Intermediate precision

The interday variation was evaluated by applying the previously mentioned procedure under linearity for the analysis of 100,120,200 $\mu g.ml^{-1}$.
The concentrations were calculated using the corresponding regression equation and the percentage recoveries and standard deviations were then calculated.

2.3.1.1.4. Robustness

The robustness as a measure of method capacity to remain unaffected by small, but deliberate, variations in method parameters was studied to test the influences of small changes in mobile phase composition, pH, wavelength of detection and flow rate.Series of system suitability parameters were calculated to test the validity of the analytical procedure.

2.3.1.2. Applications

2.3.1.2.1. Application to Hepamol tablet

The suggested procedure was applied for the determination of DL methionine in Hepamol® tablets. Samples were prepared as follows:
The contents of ten Hepamol® tablets were weighed and mixed. An amount of the powder equivalent to 25 mg of DL methionine was transferred to 250 ml beaker the powder was extracted with 10 ml water and filtered into clean 25 ml volumetric flask, washed with water and then completed to mark with water to prepare $1mg.ml^{-1}$ DL methionine stock solution.

From this solution, accurately measured volumes (1-4 ml)of DL methionine was transferred into a series of 10 ml volumetric flasks , diluted to volume with water to obtain a concentration range of (100-400 $\mu g.ml^{-1}$).
The previously mentioned procedure under linearity was then applied and the concentrations were calculated using the corresponding regression equation and the percentage recoveries were then calculated for each pharmaceutical formulation.
The validity of the proposed method was assessed by applying the standard addition technique where

known amounts of DL methionine were added to the pharmaceutical formulations. The previously mentioned procedure under linearity was then applied and the concentrations were calculated using the corresponding regression equation and the percentage recoveries were then calculated for each pharmaceutical formulation.

2.3.1.2.2. Application to soybean natural extract

200 mg of powdered soybean were accurately weighed into conical flask, HCl (9M) (12 ml) were added , mixed and hydrolysed in an oven at 110 C for 24 h. After removal from the oven, the sample was allowed to cool, centrifuged at maximum speed for 10 min to pellet debris, then the hydrolysates were diluted by adding 300 µl of distilled deionised water to a 200 µl aliquot of hydrolysate[xvii], the validity of the method was further assessed by applying standard addition technique as shown in table (4)

2.3.2. Iron thiocyanate method

The total antioxidant activity was determined according to the thiocyanate method[xviii]. In a 10 ml volumetric flask, 1 ml of DL methionine stock solution (50 mg%) was taken, 2ml of 4×10^{-2} M linoleic acid and 0.2 ml of ferrous chloride (0.1%) were added and the mixture was left at room temperature for 24 hours . The peroxide content formed was evaluated by measuring the absorbance at 500 nm after the addition of 0.1 ml of 30% ammonium thiocyanate at different time intervals and the volume was completed to 10 ml with ethanol. Control was carried out using the same procedure without the addition of DL methionine . Curve representing percentage inhibition against time was plotted.

2.3.3. Superoxide radical scavenging method

The Superoxide scavenging activity was determined using PMS-NADH system[xix]. In a 10 ml volumetric flask different aliquots (0.2-4.2 ml) of the DL methionine standard solution (1000 µg ml^{-1}) were taken, 1 ml PMS (60µM) in phosphate buffer (0.1 M,PH 7.4), 1 ml NADH (468 µM) in phosphate buffer ; 1 ml NBT (150 µM) in phosphate buffer ,each one separately was added then the volume was completed with phosphate buffer then incubated at room temperature for 5 min and the color was measured at 560 nm. Control solutions were prepared, in which phosphate buffer was used instead of the drug and the same procedure was carried out. The reagents solutions were prepared daily.[xx]

2.3.3.1. Method Validation

2.3.3.1.1. Accuracy

The previously mentioned procedure under 2.3.3. was repeated for the analysis of different concentrations of pure DL methionine and the percentage recoveries were calculated .

2.3.3.1.2. Precision

2.3.3.1.2.1. Repeatability

The intraday variation was evaluated by applying the previously mentioned procedure under 2.3.3. for the analysis of 100, 120, 200 µg.ml^{-1} DL methionine solution (n=6) and the percentage recoveries were calculated .

2.3.3.1.2.2. Intermediate precision

The interday variation was evaluated by applying the previously mentioned procedure under 2.3.3. for the analysis of 100, 120, 200 µg.ml^{-1} DL methionine solution (n=6) and the percentage recoveries were calculated.

2.3.3.1.2.3. Robustness

The robustness as a measure of method capacity to remain unaffected by small,but deliberate, variation in method parameters was carried out by changing the solvent to be potassium dihydrogen phosphate buffer, and the percentage recoveries were calculated.

2.3.3.2. Application to pharmaceutical preparation

The suggested procedure was applied for the detection of DL methionine antioxidant activity in Hepamol® tablets in presence of paracetamol . Samples were prepared as follows:

The contents of ten Hepamol® tablets were weighed and mixed. An amount of the powder equivalent to 25 mg of DL methionine was transferred to 250 ml beaker the powder was extracted with 10 ml water and filtered into clean 25 ml volumetric flask, washed with water and then completed to mark with water to prepare 1mg.ml^{-1} DL methionine stock solution. From this solution transfer different aliquots (0.2-4.2 ml) into volumetric flask (10 ml), the previously mentioned procedure 2.3.3. was then applied. Control solutions were prepared, in which phosphate buffer was used instead of the drug and the same procedure was carried out.

3. Results and Discussion:

3.1. HPLC method

In this part, HPLC method was suggested for the determination of DL methionine in pure form, in pharmaceutical formulations and in natural extracts.

Among the various modes of high performance liquid chromatography, partition chromatography is the most commonly used, with adsorption, ion exchange, gel permeation, and affinity chromatography being the principal alternative modes. Reversed phase liquid chromatography is the most commonly used [xxi] .C_{18} column was used in the study.

The specified chromatographic conditions were adjusted ; a 50 µl volume of drug solutions were injected into HPLC .Control of separation in HPLC is most commonly achieved by altering mobile phase composition, one means of varying peak resolution and retension time is changing mobile phase pH , methionine being an amino acid has both a basic amine group and an acidic carboxylic acid group.

There is an internal transfer of a hydrogen ion from the -COOH group to the -NH2 group to leave an ion with both a negative charge and a positive charge which is called a zwitterion.

When an amino acid dissolves in basic solution, the carboxyl group exists as a carboxylate anion and the α- amino group exists in its uncharged basic form.

If an amino acid dissolves in acidic solution, the -COO- group doesnot ionize, but the α- amino group is protonated [xxii] .

The phosphate buffer rendered acidic by adjusting PH at 3.2 using 1% ortho-phosphoric acid. As Rt increases when the pH of the mobile phase increases to about 7. This is because methionine becomes amphoteric and thus has more affinity for the stationary phase [xxiii] .

Several trials were carried out to obtain satisfactory separation of DL methionine in pharmaceutical formulation and in natural extract.

The chosen mobile phase was methanol: 0.05M phosphate buffer at PH 3.2 (30:70 v/v) with a flow rate 1 $ml.min^{-1}$ at wavelength 220 nm.The average retention times SD for 6 replicate injections were found to be 2.76 ± 0.43 minutes.

Calibration curve for DL methionine was constructed, representing the relationship between the calculated average peak area and the corresponding concentration, in the range of 50-350 $\mu g.ml^{-1}$ as shown in (figure 1) the regression equation was computed and was found to be

$A= 0.069C+3.5816$ $r^2=0.9986$

Where A is the peak area and C is the concentration in $\mu g.ml^{-1}$ and r is the correlation coefficient .

The accuracy of the proposed method for the assay of DL methionine in pure samples is shown in Table (1).The mean percentage recovery and standard deviation were calculated and found to be 99.85 ± 0.477.

The proposed method was successfully applied for the determination of DL methionine in pharmaceutical formulations as shown in table (2) and the validity of the method was further assessed by applying standard addition technique as shown in table (3).

Statistical comparison showed that there is no significant difference between the results obtained from the proposed method and those obtained from the Pharmacopoeial TLC method (BP)[xxiv] as shown in table (5).

The results of assay validation and system suitability testing are presented in table (6,7) . The roles for the measurements and the limits for the acceptance are given by appropriate guidelines [xxv] and pharmacopoeia [xxvi.] . From these data, one can conclude that the proposed method meet all criteria for pharmaceutical analysis.

System suitability testing (SST) for robustness study is presented in table (8). The data shows that the measured SST parameters are within the limits of acceptance given by pharmacopoeias and this ensures that the method capacity remains unaffected by small variations in method parameters and confirms that the validity of the analytical procedure is maintained whenever used.

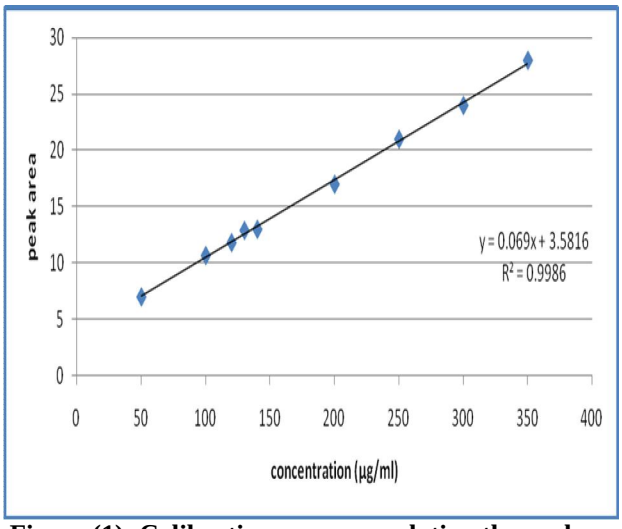

Figure (1): Calibration curve correlating the peak area to the corresponding concentration of DL methionine by the proposed HPLC method.

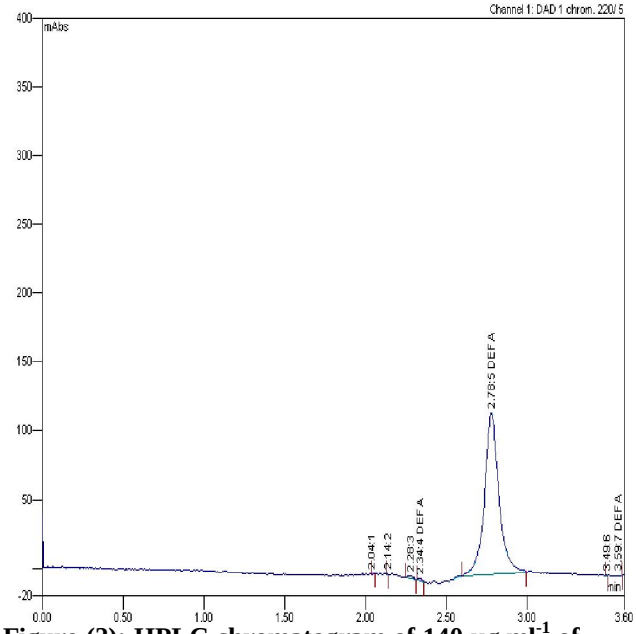

Figure (2): HPLC chromatogram of 140 µg.ml^{-1} of pure DL methionine

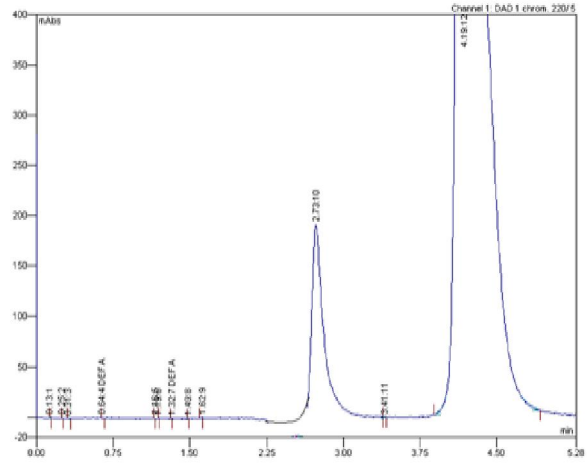

Figure (3): HPLC chromatogram of 350 µg.ml^{-1} of DL methionine in Hepamol® tablet preparation.

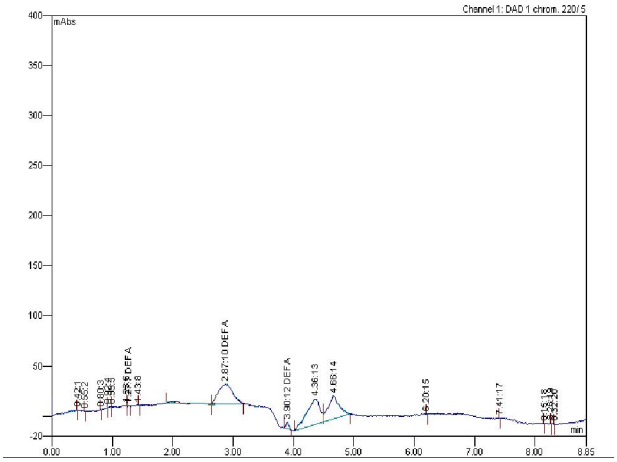

Figure (4): HPLC chromatogram of DL methionine in soyabean natural extract.

Table (1): Determination of pure samples of DL methionine by the proposed HPLC method

Taken (µg.ml^{-1})	% Recovery*
50	99.08
100	100.26
120	99.25
130	100.5
140	99.5
200	99.91
250	99.93
300	100.08
350	100.15
Mean ± SD	99.85 ± 0.477

*Average of three determinations

Table (2): Determination of DL methionine in Hepamol® tablets by the proposed HPLC method

Taken ($\mu g.ml^{-1}$)	% Recovery*
200	101.22
220	99.59
250	99.81
300	99.6
Mean ±SD	100.055± 0.678

*Average of three determinations

Table (3): Application of standard addition technique to the determination of DL methionine in Hepamol ® tablets by the proposed HPLC method

Pure added ($\mu g.ml^{-1}$)	% Recovery*
200	100.35
220	100.25
250	99.23
Mean ±SD	99.94± 0.620

*Average of three determinations

Table (4): Application of standard addition technique to the determination of DL methionine in Soyabean extract by the proposed HPLC method

Pure added ($\mu g.ml^{-1}$)	% Recovery*
195	99.80
215	99.60
245	99.10
Mean ±SD	99.50± 0.361

*Average of three determinations

Table (5): Statistical analysis of the results obtained by the proposed method and the pharmacopoeial TLC method for the analysis of DL methionine in pure form.

Parameter	Average recovery of pure DL methionine	
	The proposed HPLC method	Pharmacopoeial TLC method (BP)
Mean	99.85	100.08
SD	0.477	0.702
Variance	0.2275	0.493
n	9	5
t	-.065	--
F	2.17	--

Table (6): General characteristics of the proposed DL methionine HPLC method and validation parameters of assay:

Parameter	Value
Linearity range ($\mu g.ml^{-1}$)	50-350
Slope	0.069
Standard error of slope	0.00098
RSD slope	1.420%
Confidence limit of slope	0.067-0.071
Intercept	3.58
Standard error of intercept	0.202
Confidence limit of intercept	3.102-4.06
Correlation coefficient	0.9986
Standard error of estimation	0.279
Repeatability (SD_r)	0.296
Intermediate precision (SD_{int})	0.485

Table (7): System Suitability parameters for the analysis of DL methionine in presence of paracetamol using the proposed HPLC method

Parameter	DL methionine
Capacity factor (K')	5
Tailing factor (T)	1.14
Theortical plates (N)	2787
Selectivity (α)	1.642
Resolution (R)	3.789

Table (8): System Suitability testing data for robustness study for the analysis of DL methionine in presence of paracetamol using the proposed HPLC method:

Method parameter	Capacity factor (K')	Tailing factor (T)	Theortical plates (N)	Selectivity (α)	Resolution (R)
	DL meth	DL meth	DL meth	DL meth	DL meth
Mobile phase*	6.76	1.19	2600	1.72	2.50
PH**	7.02	1.06	2540	1.83	2.80
Flow rate***	5.56	1.15	2880	1.90	3.13
λ****	6.5	1.07	2640	1.51	2.65

* Methanol: phosphate buffer at ratios(20:80 v/v) and (40:60 v/v) were used
** PH 3.0 and 3.4 were used
*** Flow rate 0.9 $ml.min^{-1}$ and 1.1 $ml.min^{-1}$ were used
**** λ 218nm and 222nm were used

3.2. Iron thiocyanate method

During the linoleic acid peroxidation, peroxides are formed and that leads to oxidation of Fe^{2+}–Fe^{3+}. The latter ions form a complex with ammonium thiocyanate and this complex has a maximum absorbance at 500 nm. This step was

repeated every 5 h. The percentage inhibition values were calculated at this point (30 h). High absorbance indicates high linoleic acid emulsion peroxidation. Total antioxidant activity determination was performed triplicate. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

I% = [(Abs0-Abs1)/Abs0] ×100

where Abs0 was the absorbance of the control and Abs1 was the absorbance in the presence of the test compound at different concentrations [xxvii].

Lipid peroxidation contains a series of free radical-mediated chain reaction processes and is also associated with several types of biological damage. The role of free radicals and ROS is becoming increasingly recognized in the pathogenesis of many human diseases, including cancer, aging and atherosclerosis [xxviii].

The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which is the primary product of lipid oxidation.

Results obtained are shown in (figure 5), from this figure it is apparent that DL methionine has significant antioxidant activity by protection against linoleic acid oxidation and this effect decreases by time.

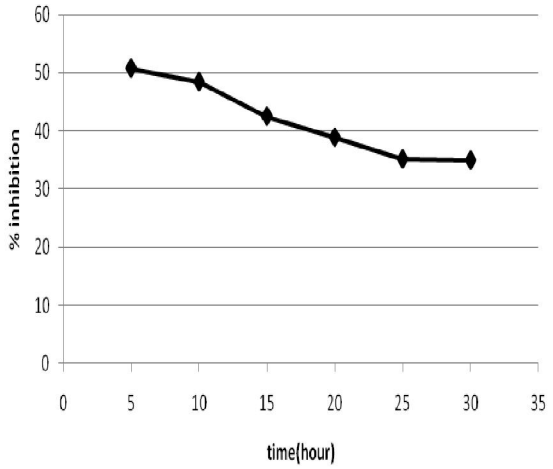

Figure (5): Percentage inhibition of linoleic acid oxidation by DL methionine

3.3. Superoxide Scavenging method

Nitro blue tetrazolium is a chemical compound composed of two tetrazole moieties, which undergoes direct reduction by superoxide free radical to form blue formazan which has absorption at wavelength 560 nm [xxix] , production of superoxide radical is occurred by reaction of phenazine methosulfate with NADH.

The influence of the sample on the scavenging of superoxide was measured by means of spectrophotometric measurement of the decrease in absorption compare to control.results are expressed as:

% scavenging = $((A_{control} - A_{sample})/A_{control}) \times 100$

The results of this method showed that DL methionine itself has a direct superoxide scavenging activity and by calculating IC_{50} value (concentration of drug required to scavenge 50% of superoxide radical) from the curve prepared from the concentrations of drug and percentage scavenging of superoxide radical ,IC_{50} of DL methionine alone was found to be 27.5 µg/ml and IC_{50} of DL methionine in presence of paracetamol doesn't significantly change but at higher concentrations % scavenging increases (figure 7), this indicates that also paracetamol acts as an antioxidant as it has a great ability to scavenge superoxide radical.

It has been stated that paracetamol has reducing and/or metal-chelating activity[xxx] also it can scavenge free radicals such as NAPQI (N-Acetyl-P-Benzoquinone Imine) in cases of acetylsalicylic acid induced gastric mucosal damage and lipid peroxidation [xxxi] .

The accuracy of the method applied on pure DL methionine is shown in table (9). The mean percentage recovery and standard deviation were calculated and found to be 100.00 ± 0.757.

The results of assay validation are presented in table (10). The rules for the measurements and the limits for the acceptance are given by appropriate pharmacopoeias, from these data one can conclude that the proposed method meet all criteria required.

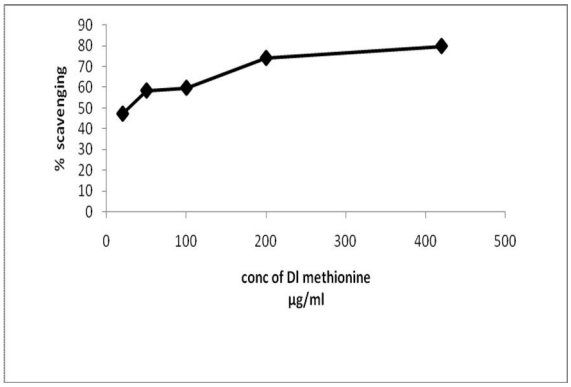

Figure (6): Scavenging effect of DL methionine on superoxide

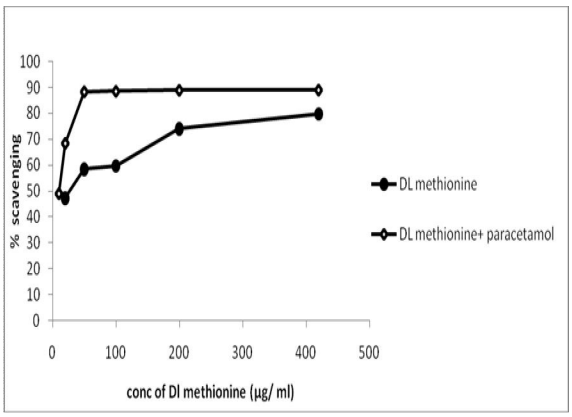

Figure (7): Scavenging effect of dl methionine and paracetamol on superoxide

Table (9): Percentage recoveries of different concentrations of pure DL methionine

Taken (µg.ml^{-1})	% Recovery*
20	99.01
50	99.6
100	100.75
200	100.75
420	99.9
Mean ± SD	100.00 ± 0.757

*Average of three determinations

Table (10): Validation parameters of assay

Parameter	Value*
Repeatability (SD_r)	0.476
Intermediate precision (SD_{int})	0.8919
Robustness	0.378

*Average of three determinations

4. Conclusion

The proposed HPLC method is successfully applicable for the analysis of DL methionine in its pure form, in its multiingredient formula with paracetamol (Hepamol tablet) and in soybean extract. The method was evaluated and validation procedure was proposed.

The proposed HPLC method is accurate, precise and specific over the specified range and thus can be used for routine analysis of DL methionine in quality control laboratories.

Also it is clearly indicated that DL methionine itself has an antioxidant activity by scavenging superoxide radical and by protection against linoleic acid oxidation; moreover the addition of paracetamol to DL methionine increases the superoxide scavenging activity.

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