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BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF EDARAVONE BY RP-HPLC: APPLICATION TO HUMAN CLINICAL STUDIES

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ABSTRACT
The objective of the study is to develop and validate bio-analytical method of edaravone using drug free human plasma. The present method is using optimized parameters such as wavelength, mobile phase and ratio, flow rate, pH and buffer strength, among all the above mentioned parameters, the best separation of Edaravone is achieved using 5mM ammonium acetate (pH 4.5) and acetonitrile in the ratio of 40:60 with 0.9 ml/min flow rate. The plasma recovery was shown 89.56% in human drug free plasma. The linearity of the method was shown in the range of 500ng/ml to 7500ng/ml. The minimum quantifiable concentration of edaravone was determined to be 300ng/ml. This method is applicable to bioavailability, bioequivalence and pharmacokinetic studies.

Keywords: Edaravone, Bio-analytical method development and validation, HPLC, Pharmacokinetic studies.

INTRODUCTION
Edaravone is 5-methyl-2-phenyl-2, 4-dihydro-3H-pyrazol-3-one is a pyrazole derivative appears as white to off white crystalline powder [1, 2]. The drug is freely soluble in HPLC water. Edaravone is a five-membered pyrazole ring, weak base with pKa values of 7 [3]. Edaravone melts at 127-131ºC. Edaravone is a neuroprotective agent used for aiding neurological recovery following acute brain ischemia and subsequent cerebral infarction [4]. It acts as a potent antioxidant and strongly scavenges free radicals, protecting against oxidative stress and neuronal apoptosis [5-7]. Many literature survey shows that the analytical methods of measuring is limited, edaravone plasma concentrations measurements have been done only by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and gas chromatography with mass spectrometry (GC-MS) [8-10]. To the best of our knowledge no reports were found for the validation of edaravone in drug free human plasma. The objective of this study was to develop and validate an assay for the edaravone using RP-HPLC.

EXPERIMENTAL
Reagent and materials:
Edaravone were obtained from Merck (Germany). HPLC–grade acetonitrile, AR grade ammonium acetate, orthophosphoric acids were purchased from Bendosen, Penang (Malaysia) deionised water was produced by a Direct Millipore water system. The human blank plasma was supplied by the local hospital, Sungai petani, Malaysia.

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Instrumentation:
HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan).

Standard solutions:
A stock solution of edaravone was prepared by dissolving the appropriate amount of methanol in order to obtain a final concentration of 1.0mg/ml solution. Label and store the solution in a below 8°C, Working standard solutions were prepared freshly with an appropriate dilution.

Chromatographic technique:
All chromatographic experiments were carried out in the isocratic mode. The Thermo C_{18} (250 x 4.6 mm i.d.5µ). The mobile phase consisted of a mixture of 5mM Ammonium acetate and acetonitrile (60:40%v/v). The flow rate was 0.9 ml/min and the volume injected 25µl using Auto injector. The standard chromatogram is presented in Figure 1.

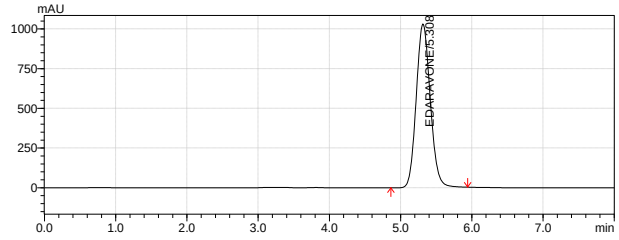

Figure-1: The standard chromatogram of Edaravone

Calibration curve solutions used for method validation:

Six solutions of edaravone were prepared by diluting the stock solution with the mobile phase to reach concentrations ranging from 500ng/ml to 7500ng/ml. These solutions were then used to spike plasma samples for calibration curve.

Sample preparation:

At the time of validation, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. A volume of 0.5 ml of sample was pipetted into 2.0ml centrifuge tube with this 0.5ml of precipitating agent (10% Trichloro acetic acid) was added. The resulting solution was vortexes for 5 minutes and centrifuged at 5000 rpm for 7mins. Supernatants from the above solutions used for the analysis. The sample chromatogram is presented in Figure 2.

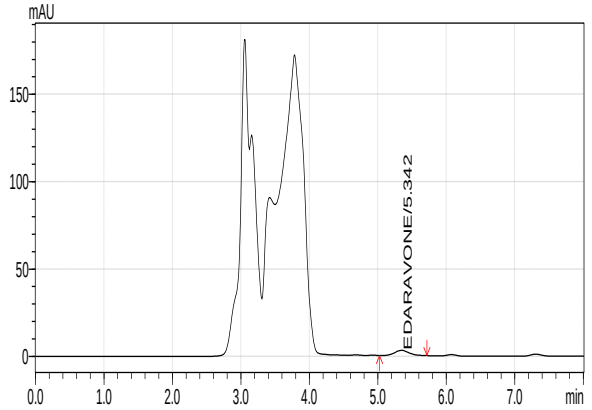

Figure-2: The sample chromatogram of Edaravone

VALIDATION

Selectivity:

The Selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing edaravone and internal standards with those obtained from blank (Figure 3).

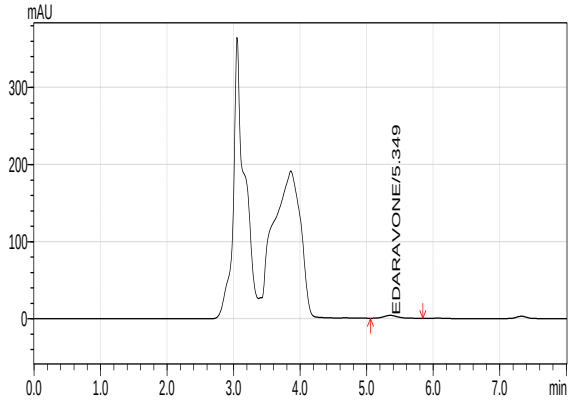

Figure-3: Blank chromatogram of Edaravone

Sensitivity:

Determine sensitivity in terms of LLOQ 'lower limit of Quantification 'where the response of LLOQ should be at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte.

Linearity Range:

The linearity different concentrations of standard solutions were prepared to contain 500ng/ml to 7500ng/ml of edaravone. These solutions were analysed and the peak areas were calculated. The calibration curve was plotted using response factor Vs concentration of the standard solutions. The Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighing and statistical tests for goodness of fit.

Precision and Accuracy:

The precision of the method was determined by intra-day precision and inter-day precision. The intra-day precision was evaluated by analysis of blank plasma sample containing edaravone three different concentrations of LQC, MQC and HQC using nine replicate determinations for three occasions. The inter-day precision was similarly evaluated over two-week period.

Standard stock solution stability:

Room temperature stock solution stability was carried out at 0, 3 and 8 hours. by injecting four replicates of prepared stock dilutions of edaravone equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of edaravone at 3 and 8 hrs was carried out against the zero hour value.

Refrigerated stock solution stability:

Refrigerated stock solution stability was carried out at 7, 14 and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration.

Freeze thaw stability, Short-term stability and long-term stability:

The stability studies of plasma samples spiked with edaravone were subjected to three Freeze - thaw cycles, Short-term stability at room temperature for 3 hrs and long term stability at – 70^{o} C over four weeks. In addition, stability of standard solutions was performed at room temperature for 6 hrs. and freeze condition for four weeks. The stability of

triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short-term samples spiked with edaravone was kept at room temperature for 1to 3 hrs. before extraction. The plasma samples of the long-term stability were stored in the freezer at -70^0 C until the time of analysis.

Partial volume analysis:

Five sets of middle and high concentration using 25% and 50% processing volume were processed and were injected along with calibration curve standards and the concentrations were calculated using appropriate dilution factor.

RESULT AND DISCUSSIONS

The no endogenous source of interference was observed at the retention times of the analytes. The limit of reliable quantitation was set at the concentration of the LOQ QC, 500ng/ml, for edaravone. At the LOQ QC concentration, the precision and accuracy for edaravone is 1.07 (Table 1).

The calibration curves showed linear response over the range of concentration used in the assay procedure. A regression equation with a weighing factor of 1/concentration^2 was applied. The inter and intra-day precision and accuracy results showed in the Table 2 and 3 respectively. Intra-day precision for edaravone ranged from 0.07% to 0.82% and the intra-day accuracy for edaravone ranged from 99.39% to 99.89%. Inter-day precision for edaravone ranged from 0.01% to 0.10% and the inter-day accuracy for edaravone ranged from 99.70% to 99.98%. In the stock solution stability, freeze thaw stability, Short term stability result showed in the Table 4. In partial volume analysis the within batch precision and accuracy of edaravone for a processing volume of 25% ranged from 0.02 % to 0.99% and 99.1% to 99.97%, Similarly the within batch precision and accuracy of edaravone for a processing volume of 50% ranged from 0.01% to 0.04% and 99.79% to 99.97%. (Table 5). Long term stability studies, no tendency of degradation stability result showed in the Table 6.

Table-1: Sensitivity

Nominal concentration (ng/ml)	500
Mean	495.55
SD	5.34
% CV	1.07
% Nominal	99.11
N	5

Table- 2: Intra-day precision and accuracy

Nominal Concentration (ng/ml)	500	3500	7500
Mean	496.96	3490.92	7491.86
SD	4.12	5.57	5.27
% CV	0.82	0.15	0.07
% Nominal	99.39	99.74	99.89
N	5	5	5

Table- 3: Inter-day precision and accuracy

Nominal Concentration (ng/ml)	500	3500	7500
Mean	498.50	3498.48	7498.76
SD	0.54	0.95	0.67
% CV	0.10	0.02	0.01
% Nominal	99.70	99.95	99.98
N	5	5	5

Table- 4: Stock solution Stability of edaravone

Room Temperature	
No. of Hours	% Initial
0	100.2
3	105.4
7	107.8

Table- 5: Partial Volume Analysis

Nominal	Half Volume		Quarter Volume	
Concentration ng/ml	500	3500	500	3500
	LQC	MQC	LQC	MQC
Mean	495.81	3499.16	498.97	3498.96
SD	4.9	0.90	0.24	0.43
% CV	0.99	0.02	0.04	0.01
% Nominal	99.10	99.97	99.79	99.97
N	5	5	5	5

Table- 6: Stability Studies

Nominal Concentration (ng/ml) (N = 5)	Concentration found (ng/ml)	Precision (%)	Accuracy (%)
Short-term stability			
for 3 hrs in plasma			
500	499.10	0.15	99.81
3500	3499.43	0.01	99.98
Three freeze-thaw cycles			
500	498.81	0.23	99.76
3500	3498.60	0.01	99.96
Long-term stability			
for 30 days -50 ◦C			
500	497.88	0.19	99.57
3500	3490.05	0.02	99.71

CONCLUSION

The HPLC-UV method for the quantitation of edaravone in human plasma was developed and fully validated as per FDA guidelines. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (7 min) and lower sample requirements. Hence, this method is useful for single and multiple ascending dose studies in human subjects and pharmacokinetic profile.

The current method has shown acceptable precision and adequate sensitivity for the quantification of edaravone in drug free human plasma. The developed method has excellent sensitivity, reproducibility and specificity. The method has been successfully used to provide the bio-analytical study of edaravone in drug free human plasma. The developed assay showed acceptable precision, accuracy, linearity, stability, and specificity.

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