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A VALIDATED, SPECIFIC STABILITY-INDICATING RP- LC METHOD FOR CITICOLINE AND ITS RELATED SUBSTANCES IN ORAL DROPS

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ABSTRACT

A validated, specific, stability indicating reversed-phase liquid chromatographic method has been developed for quantitative analysis of citicoline and its related substances in oral drops formulation. Forced degradation studies were performed using acidic, basic and oxidizing conditions, and thermal and photolytic stress, to show the stability indicating power of the method. The method was optimized by analysis of the samples generated during the forced degradation studies and sample solutions spiked with the impurities. Good resolution between the analyte peak and peaks corresponding to process related impurities and degradation products was achieved on a Whatman Partisil SAX C_{18} (250X4.6mm, 10µm) column with a mobile phase constituted of phosphate buffer and acetonitrile (40:60 % v/v) and further comprises of 1gm of 1-hexane sulphonic acid sodium salt. The pH of the mobile phase was adjusted to 3.4 with ortho phosphoric acid. Detection was performed at 280 nm. Citicoline sodium was subjected to stress conditions of hydrolysis (acid, base), oxidation, photolysis and thermal degradations. The method was validated in accordance with ICH guidelines and the validation data showed that the assay is sensitive, specific and reproducible for the determination of citicoline in the presence of related substances and degradation products.

Key words: Citicoline, related substances, column liquid chromatography, degradation.

INTRODUCTION

Citicoline (cytosine-1B-D-ribofuranosyl-5'-diphosphocholine) is the generic name of synthetic CDP-choline (cytidine diphosphate choline), organic molecule produced endogenously and found in all living cells. CDP-choline a precursor for the synthesis of phospholipids that are essential constituents of cell membranes, including phosphotidyl choline, phosphotidyl serine and phosphotidyl ethanolamine. Because cell membranes have a very high turnover rate, these phospholipids must be continuously synthesized to ensure adequate function of cells. Citicoline is often called as brain nutrient because it increases levels of several neurotransmitters including acetylcholine, dopamine and nor adrenaline; helps maintain the integrity of neuronal cell membranes; and increases energy production in the frontal cortex. The scientific name for citicoline is 5-diphosphocholine. Citicoline is degraded to uridine and choline during intestinal absorption [1]. These two compounds then pass through the blood brain barrier to reconstitute citicoline in the brain [2].

The literature survey reveals that very few methods were reported for the estimation of citicoline sodium in plasma, serum and pharmaceutical preparation by LC. Gu, S.Q reported a method for the determination of citicoline sodium and its injection by HPLC [3]. Mirakor et al., described a sensitive high performance liquid chromatography assay method for citicoline in formulation dosage form [4]. Raveendra et al., reported a stability indicating LC method for the determination of citicoline sodium in injection formulation [5]. None of the reported analytical procedures describe a method for the determination of citicoline in oral drops formulation in the presence related substances and degradation products generated from forced degradation studies. This research was conducted to develop a suitable stability indicating single LC method for analysis of citicoline and its related substances namely imp A (cytidine), imp B (uridine), imp C (Cytidine mono phosphate morfolidato), imp D (cytidine-5'-monophosphate free acid), imp E (Uridine-5'-monophosphate disodium salt) and imp F (Uridine diphosphate colina).

MATERIALS AND METHODS

All the reagents were of analytical-reagent or HPLC grade unless stated otherwise. Milli-Q-water was used throughout the experiment. Di basic potassium phosphate, 1-hexane sulphonic acid sodium salt, phosphoric acid and acetonitrile (Spectrochem, Mumbai, India), hydrogen peroxide and hydrochloric acid(S.D. Fine Chem., Mumbai, India) and sodium hydroxide (Central drug house Pvt. Ltd, Mumbai, India) were used. Citicoline sodium dihydrate standard was obtained from strides arco laboratories (Bangalore, India).Citicoline sodium oral drops (100 mg mL^{-1}) formulation was purchased from the local pharmacy.

Instrumentation

The HPLC system used was an Agilent 1200 series comprised of degasser, quaternary pump, auto sampler, thermo stated column compartment, photo diode array detection and the system was controlled through Chemstation software. Analytical column used for this method is Whatman Partisil SAX C_{18} (250X4.6mm, 10µm).

Mobile phase preparation

First the phosphate buffer was prepared by dissolving 6.8 gm of dibasic potassium phosphate in 100 mL of water. Then the buffer solution was mixed with acetonitrile in the ratio 400:600 and then 1gm of 1-hexane sulphonic acid sodium salt was added. pH of the mobile phase solution was adjusted to 3.4 with ortho phosphoric acid.

Standard Preparation

Standard stock solution was prepared by dissolving 28 mg of citicoline sodium dihydrate (equivalent to 25mg of citicoline) in sufficient amount of water in a 100 mL volumetric flask and diluted up to the mark with water. 2.0 mL of the above solution was further diluted to 50.0 mL with water.

Sample Preparation

An accurately measured amount of sample, equivalent to 200 mg of citicoline (2.0 mL) was transferred to a 200 mL volumetric flask and make the volume with water.

Chromatographic conditions

The mobile phase was filtered through 0.45 µm, PVDF membrane filter and degassed using vacuum before delivering into the system. For analysis of forced degradation samples, the photo diode array detection was used in scan mode with a scan range of 200-400 nm. The peak homogeneity was expressed in terms of peak purity and was obtained directly from the special analysis report obtained using the above mentioned software. The chromatographic conditions used for the analysis were given below.

Column	:	Whatman Partisil SAX C_{18} (250X4.6mm, 10µm)
Wavelength	:	280 nm
Injection volume	:	10 µl
Flow rate	:	1.0 mL min^{-1}
Column temperature	:	35^0C

Specificity/ Selectivity

Specificity is the ability of the method to asses unequivocally the analyte in the presence of components, which may be expected to be present. Typically these might include impurities, degradation products, matrix, etc. The specificity of the developed method for citicoline sodium was carried out by injecting diluent, placebo, citicoline sodium and impurities. The peaks of placebo and impurities were not interfering with the citicoline sodium peak.

Procedure for forced degradation study of citicoline sodium [6, 7]

Acidic degradation

A measured amount of sample equivalent to about 200 mg of citicoline (2.0 mL) was transferred to a 200 mL volumetric flask, and then 2.0 mL of 0.1N HCl was added and kept at 80^0C about 4 hrs in water bath, the solution was allowed to attain room temperature. Then the solution was neutralized by 0.1N NaOH and diluted to volume with water and repeated the same with 1.0N HCl to get the accurate value.

Alkali degradation

A measured amount of sample equivalent to about 200 mg of citicoline (2.0 mL) was transferred to a 200 mL volumetric flask, and then 2.0 mL of 0.1N NaOH was added and kept at 80^0C about 4 hrs in water bath, the solution was allowed to attain room temperature. Then the solution was neutralized by 0.1N HCl and diluted to volume with water. Repeated the same with 1.0N NaOH.

Oxidative degradation

A measured amount of sample equivalent to about 200 mg of citicoline (2.0 mL) was transferred to a 200 mL volumetric flask, then 2.0 mL of 3% H_2O_2 was added and kept at 80^0C about 4 hrs in water bath, the solution was allowed to attain room temperature and made up the volume with water.

Thermal degradation

A measured amount of sample equivalent to about 200 mg of citicoline (2.0 mL) was transferred to a 200 mL volumetric flask, and then 2.0 mL of 0.1N HCl was added and kept at 80^0C in vacuum oven for 4 hrs, the solution was allowed to attain room temperature. Then made up the volume with water.

UV degradation

The sample was exposed to UV short (254 nm) and UV long (365 nm) light for 8 hrs. A measured amount of UV light exposed sample equivalent to about 200 mg of citicoline (2.0 mL) was transferred to a 200 mL volumetric flask and made up the volume with water.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

The primary target in developing this stability indicating LC method was to achieve the resolution between citicoline and its related substances. To achieve the separation of related substances, stationary phase of C_{18} and a combination of mobile phase phosphate buffer with methanol and acetonitrile were used. The separation of degradation products and citicoline sodium was achieved on Whatman Partisil SAX C_{18} column and a mobile phase composed of phosphate buffer and acetonitrile in the ratio 400:600 and 1gm of

1-hexane sulphonic acid sodium salt. pH of the mobile phase was adjusted to 3.4 with ortho phosphoric acid. Mobile phase flow rate was maintained at 1.0 mL min^{-1} and eluent were monitored at 280 nm. A 10 µl of sample was injected using a fixed loop. The forced degradation study showed that the method was highly specific and all the related substances were well resolved from the main peak (Fig. 1). Placebo, citicoline and sample as such were also injected and the chromatograms given in Fig.2.

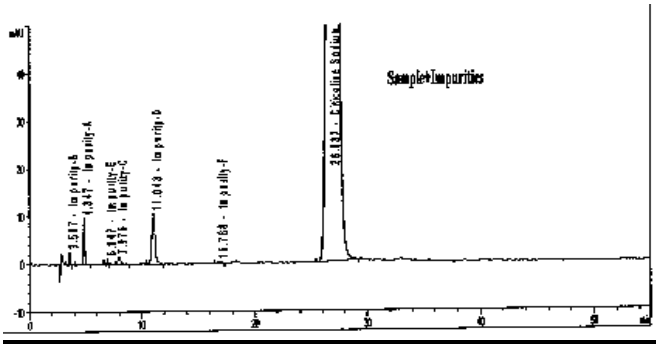

Fig.1: Chromatogram of citicoline sodium spiked with impurities

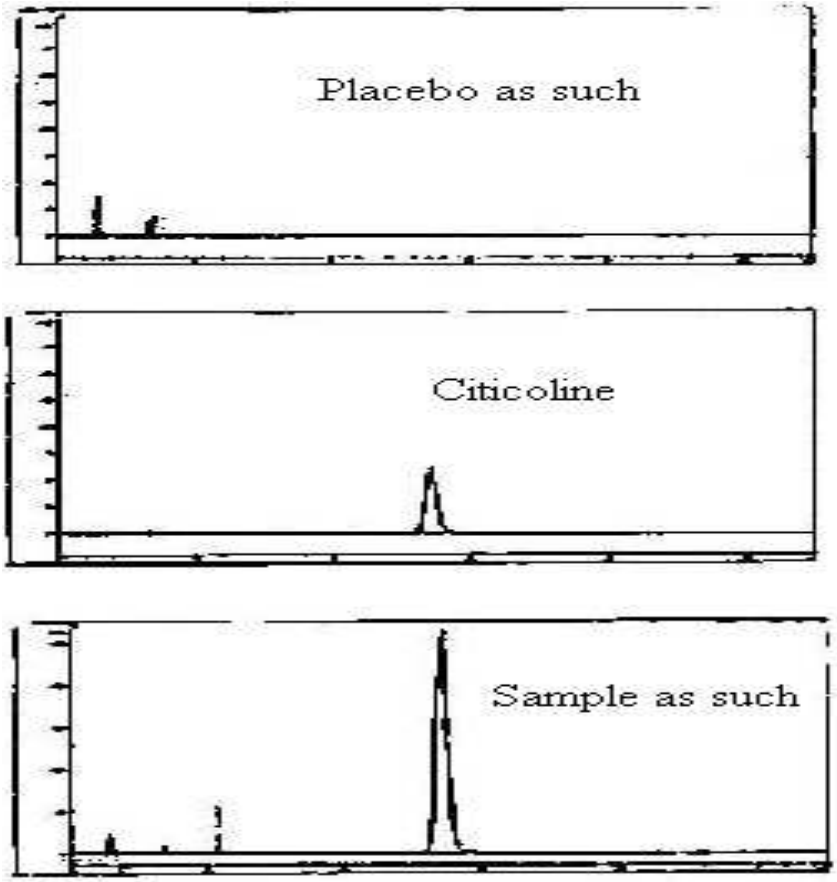

Fig. 2: Chromatograms of Placebo, citicoline and sample as such

Results of forced degradation Experiments

The degradation behavior of citicoline sodium in various stress conditions was shown in Fig.1.Peak purity results greater than 990 indicate that the citicoline sodium peak is homogeneous in all stress conditions tested. The results were shown in Table 1.

Table 1: Peak purity data for citicoline sodium

S.No	Condition	Purity Factor
1	0.1N HCl	998.91
2	1.0N HCl	999.90
3	0.1N NaOH	999.91
4	1.0N NaOH	998.90
5	Sunlight	998.66
6	UV-light	999.54
7	Dry Heat	999.64
8	3% H2O2	998.99

Method validation

The optimized chromatographic conditions were validated by evaluating specificity, linearity, precision, accuracy, robustness and system suitability in accordance with ICH guidelines Q2A (R1).

Linearity

The linearity of detector response to different concentrations of impurities was studied in the range of 0.5– 10.0 µg mL^{-1} at ten different levels. Similarly, the linearity of citicoline sodium was studied by preparing standard solutions at ten different levels ranging from 10 to 300 µg mL^{-1}. The data were subjected to statistical analysis using a linear regression model; the regression equations and coefficients (r2) are given in Table 2. The results indicated good linearity.

Table 2: Linearity Data

Sample	Range (µg mL^{-1})	Regression equation	r^2
Citicoline	10-300	y = 79279x-2116	0.9999
Imp-A	0.5-10	y = 75378x+5378	0.9944
Imp-B	0.5-10	y = 57152x+4463	0.9932
Imp-C	0.5-10	y = 49751x+1136	0.9870
Imp-D	0.5-10	y = 23304x+1710	0.9867
Imp-E	0.5-10	y = 44653x-8621	0.9893
Imp-F	0.5-10	y = 22378x-2978	0.9931

Precision

Citicoline sodium was spiked with 0.1% w/w of each impurity and the precision of the method was tested by six (n = 6) injections of the above solution. The RSD of retention time (tR) and peak areas were in the range of 0.1–0.60%. The precision in determination of citicoline sodium and impurities was studied by repeatability, intermediate precision, and reproducibility (ruggedness). Repeatability is the intraday variation in assay obtained at different concentration levels, and is expressed in terms of RSD calculated for each day. The RSD values were found to be below 0.71% (for impurities) and 0.61% (CSD), indicating a good repeatability. The intermediate precision is the interday variations calculated for five concentration levels for 6 days and expressed in terms of % RSD values. At each concentration level, the % RSD values were below 9.41 (for impurities) and 0.61% (CSD), indicating a good intermediate precision. The ruggedness of the method was determined by analyzing the same samples in triplicate for 2 days by another instrument by a different analyst with different lots of reagents and columns. The data obtained were within 2.0% RSD.

Accuracy

The recoveries of impurities A, B, C, D, E, and F were determined by spiking known quantity of each impurity at 50,100,200 and 300% of specification level into the sample. The spiked samples were analyzed in triplicate for each level. The recovery range and RSD for all impurities were found to be 91.7–107.6 and 0.1–2.7%, respectively.

Robustness

It is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To determine the robustness of the developed method, typical variations in analytical conditions were tested. Influence of flow rate, mobile phase composition, pH and change in column temperature were studied. All known and unknown impurities were well separated from each other in sample spiked with impurities. The theoretical plate for

citicoline sodium peak was not less than 2000 in diluted standard preparation and the ratios between the areas of the duplication injections of diluted standard preparation were in between 0.95 and 1.05. The results were shown in the Table 3.

Table 3: Robustness study of citicoline sodium

		Ratio between the areas of duplication injections of diluted standard preparations	**Theoretical plate**
Original Condition		1.01	4969
Flow change	-0.2 mL min^{-1}	0.99	5772
	+0.2 mL min^{-1}	1.01	4576
Change in column temperature	- 5^0C	0.99	4753
	+5^0C	1.00	5283
pH	-0.2 units	0.99	5114
	+0.2 units	0.99	5041
Change in organic phase	-2.0 %	0.99	4887
	+2.0 %	0.99	5018

CONCLUSIONS

A simple isocratic RP-HPLC method was developed and validated for determining citicoline sodium and its process related substances in oral drops. Attempts were made to separate citicoline sodium and its process-related impurities on different commercial C18 columns. Studying the effects of organic modifier and concentration and pH of buffer chromatographic conditions were optimized. The developed method was found to be selective, precise, linear, accurate, and reproducible in determining the citicoline sodium and its potential impurities.

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