HPLC DETERMINATION OF ASPARTAME IN TABLETOP SWEETENERS BY PRECOLUMN DERIVATIZATION USING 2, 4-DINITROFLUOROBENZENE

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ABSTRACT

A new, simple normal phase high performance liquid chromatographic method was developed for the analysis of aspartame in tabletop sweeteners. The method is based on the derivatization of aspartame with 2, 4-dinitrofluorobenzene followed by detection at 332 nm. Separation was carried on silica column with hexane: ethyl acetate (60: 40, V/V) as mobile phase (pH was adjusted to 4.5 using 1% acetic acid) with a flow rate of 1 ml/min. The limit of detection (LOD) and limit of quantification (LOQ) of this method was found to be 1 ng and 4 ng respectively. The method was found to be linear in the concentration range of 20-180 ng. The recovery values of this method were found to be better than 97%.

Keywords: Aspartame, 2, 4-dinitrofluorobenzene, Normal phase, HPLC, Derivatization, Tabletop sweetener.

INTRODUCTION

Aspartame is a nutritive intense sweetener produced by combining the amino acids L-phenylalanine and L-aspartic acid by a methyl ester link (Fig 1). Its chemical name is (35)-3-amino-N [alpha-S-alphamethoxycarbonylphenythyl] succinic acid but it is often described by its synonym L-aspartyl-L-phenylalanine methyl ester. It was discovered by J.D. Schlatter in 1965 in the laboratories of G.D. Searle. Aspartame has a clean sweet taste and is approximately 18-200 times the sweetness of sucrose [1].
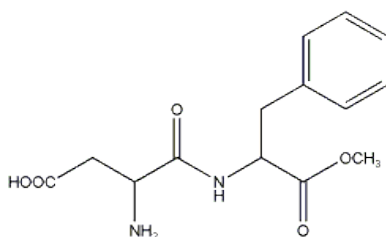

Fig. 1: Structure of Aspartame

Aspartame has been the subject of several controversies, since its initial approval by the U.S. Food and Drug Administration (FDA) in 1974. Upon ingestion, aspartame breaks down into natural residual components, including aspartic acid, phenylalanine, methanol [2]. Aspartame is probably the most rigorously tested food ingredient to date. Prior to its approval in major market, aspartame and its breakdown products underwent extensive tests to validate its safety [3]. Significantly controlled peer reviewed studies have consistently failed to produce evidence of a casual effect between aspartame consumption and adverse health events. Additional reviews of aspartame safety covering studies done pre- and post-approval have been carried out by regulatory bodies. These have confirmed that aspartame is a safe and thoroughly tested food ingredient [4]. Food and Drug Administration (FDA) have assigned aspartame an ADI of 50 mg/kg bw [5].

Aspartame was analyzed by using thin layer chromatography (TLC) using ninhydrin as a visualization reagent [6]. Several capillary electrophoresis (CE) methods were reported for analysis of aspartame with UV detection at 254 nm [7-9]. Flow injection analysis (FIA) of aspartame was performed using ninhydrin as colorimetric reagent [10]. A bienzymatic electrode has been utilized for electrochemical analysis of aspartame [11]. Several spectrofluorometric methods involving ninhydrin [12] and enzymatic method using α-chymotrypsin-alcohol oxidase were also reported for the analysis of aspartame [13]. Among the all methods described for the analysis of aspartame high performance liquid chromatography (HPLC) is the most popular technique. Several reverse phase HPLC methods with UV detection at 254 nm were reported by several researchers [14-17]. The liquid chromatographic methods with chemical derivatization using naphthalene-2, 3-dicarboxyaldehyde [18] and fluorescamine [19] were reported showing good sensitivity. But the main drawback of these methods is they require lengthy and complicated derivatization procedure.

In this paper we describe a new simple and sensitive normal phase HPLC method based on the pre-column derivatization of aspartame using 2,4-dinitrofluorobenzene with detection at 332 nm. The developed method was also compared with official HPLC [20] method in terms of recovery.

MATERIALS AND METHODS

Chemicals and Reagents

Aspartame was purchased from Sigma-Aldrich (India), 2,4-dinitrofluorobenzene (DNFB), HPLC grade chloroform and ethyl acetate were purchased from Qualigens (India). Other chemicals used were of analytical reagent grade and purchased from Sd. fine chemicals (India). Water used for preparation of reagents was of double distilled water. Tabletop sweeteners containing aspartame were purchased from local market.

Apparatus

HPLC system (Waters) consisting of a 600E controller pump, a 717 plus auto sampler, 2996 PDA detector and an inline-degasser. Millinium32 software for data processing and silica (Waters, Spherisorb 5um silica, 4.6 x 250 mm) analytical column was used for separation. The mobile phase consisted of (hexane: ethyl acetate) (60:40, v/v, pH adjusted to 4.5 using 1% acetic acid), with a flow rate of 1 mL/min. The analysis was carried out at ambient temperature.

Standard solution

Stock solution of aspartame (0.2 mg / mL) was prepared by dissolving in water. For drawing calibration curve five different concentrations in linearity range were prepared by diluting stock solution.

Sample preparation

Accurate weight of powdered tablets equivalent to 10 mg of drug was transferred quantitatively to 100 mL volumetric flask with the aid of 50 mL of water. The content of the flask was sonicated for 10 minutes in ultrasonic bath the flasks were filled upto the mark with water and filtered before derivatization.

Derivatization

In a 10 mL screw cap test tube 1 mL of each standard solution was added. This was followed by addition of 1 mL of 0.2 mol / L sodium bicarbonate (NaHCO$_2$) solution and 1 mL of 1% DNFB (a 1 mL portion of DNFB was dissolved in 100 mL of 1,4-dioxane). These test tubes were then placed on a water bath (60°C) for 40 minutes. All derivatization reactions were stopped by addition of 0.5 mL of 1 mol / L HCL. The resulting dinitrophenylamine (DNP) derivative was extracted with 5 mL ethyl acetate and made up to 10 mL with mobile phase. All the samples were treated in the same manner as described above for standard. To avoid interference from components soluble in ethyl acetate, all the samples were pre-extracted with 5 mL of ethyl acetate before derivatization.

Ten (10) µL of each standard and sample were injected into the HPLC system.

Method Validation

Preliminary validation of the method was performed by checking the linearity, precision, recovery, detection and quantification limits, and repeatability.

Limits of Detection and Determination

Limits of detection (LOD) and limits of quantification (LOQ) of the method were determined by injecting standard serial dilutions (made from stock solution of 0.2 mg / mL) of progressively decreasing concentrations run on HPLC. The LOQ and LOD were the concentrations for which signal-to-noise ratio (S/N) was 10:1 and 3:1 respectively[21].

Linearity

For linearity checking Stock solution (0.2 mg/mL) was further diluted to give the final concentration of 0.1 µg/mL. And these solutions were injected into the HPLC system and linear curve was drawn by taking the resultants peak areas and concentration.

Precision

The precision of the method was evaluated on the basis of analyzing the three different concentrations (50 µg/mL, 100 µg/mL, 150 µg/mL) of aspartame in the linearity range for repeating three times.

Recovery/Accuracy

The accuracy of the method was expressed as the percentage recovery of each

component. Recovery studies were carried out by standard addition method where three different concentrations (50 µg/mL, 100 µg/mL, 150 µg/mL) of above said components. Recovery study was carried out on tabletop sweeteners samples.

Repeatability

The consistency of the results for the same analyte samples were checked by repeating the experiment 6 times per day (intraday) and consecutively for 3 days (interday). The standard deviation of the repeated recovery values was calculated.

Robustness

Robustness is a measure of a method's immunity to small but deliberate variations in the conditions used[22]. Chloroform, ethyl acetate ratio and flow rate were deliberately changed and the effects were monitored.

RESULTS AND DISCUSSION

Aspartame is a dipeptide composed of two amino acids, L-aspartic acid and the methyl ester of L-phenylalanine. The proposed method is based upon the derivatization of aspartame with 2, 4-dinitrofluorobenzene (DNFB) (Sanger's reagent) (Fig 2). 2, 4-dinitrofluorobenzene reacts with the amino group of the amino acids to form the dinitrophenyl-derivative [23]. This property was used for derivatization of aspartame where DNFB reacts with amino group of aspartame to form the dinitrophenyl-aspartame (DNP-ASP) derivative which was than recovered and analyzed through HPLC.

Optimization of conditions for derivatization reaction

Both amino acids and amines could be derivatized by DNFB in weakly basic solutions, but the need for time / temperature control would vary according to the characteristic of compounds. An experimental scheme was designed with three factors i.e., concentration of sodium bicarbonate, reaction temperature and reaction time on the maximum peak area of the derivatized aspartame which was extracted in ethyl acetate (Table-1).
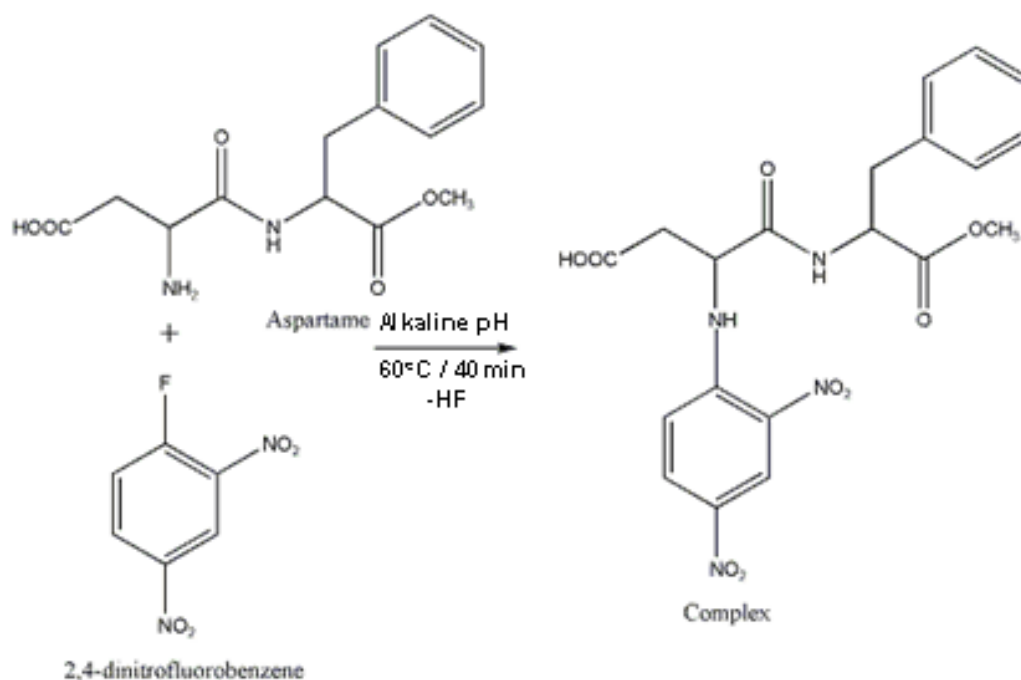

Fig. 2: Scheme showing the reaction of aspartame with 2,4-dinitrofluorobenzene
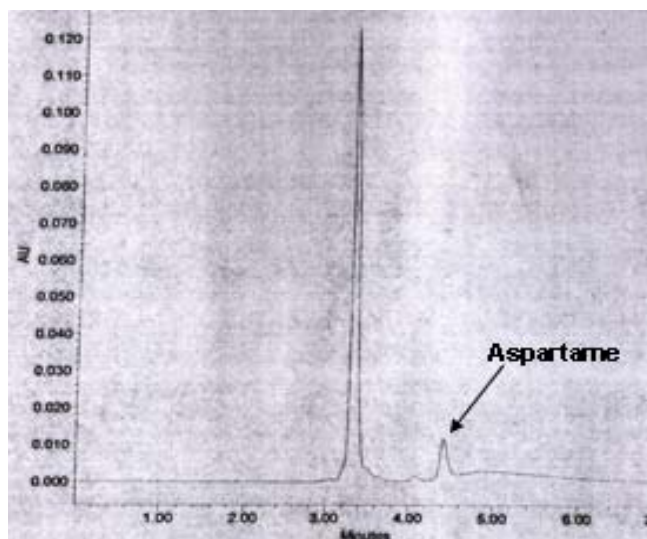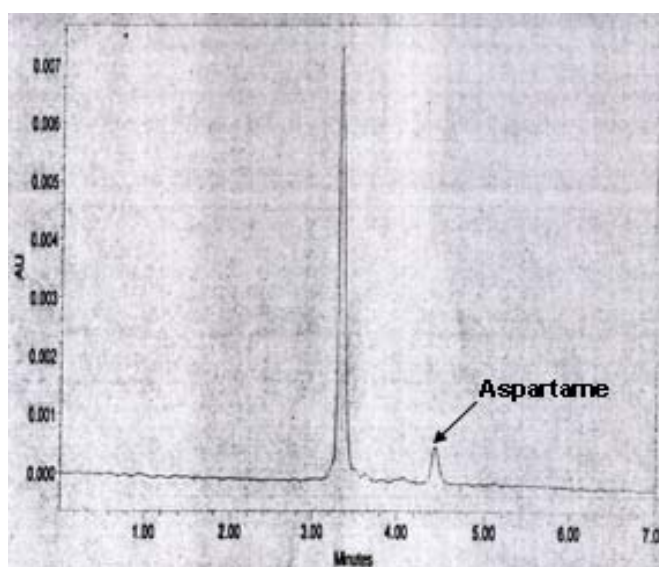
Fig. 3.1: Chromatogram obtained for standard aspartame

Fig. 3.2: Chromatogram obtained for tabletop sweetener sample

Fig. 3: Chromatogram obtained for proposed method

Table1: Experimental scheme showing the effects of different factors on derivatization reactions and the resulting peak area values

Treatment	NaHCO$_3$ (mol/L)	Temperature (C^o)	Time (min)	Peak area
1.	0.0	40	20	-
2.	0.0	50	30	-
3.	0.0	60	40	-
4.	0.0	70	50	-
5.	0.1	40	20	97123
6.	0.1	50	30	98542
7.	0.1	60	40	98702
8.	0.1	70	50	98834
9.	0.2	40	20	99352
10.	0.2	50	30	99578
11.	0.2	60	40	99949
12.	0.2	70	50	99159
13.	0.4	40	20	97198
14.	0.4	50	30	97463
15.	0.4	60	40	98198
16.	0.4	70	50	98356

Table 2: Showing the values of LOD, LOQ, linearity and regression value

S. No	Parameters	Value
1.	Limit of detection (LOD)	1 ng
2.	Limit of quantification (LOQ)	4 ng
3.	Linearity range	20 – 180 ng
4.	Intercept (c)	289.26
5.	Slope (m)	928.92
6.	Correlation coefficient (R^2)	0.9998
7.	Regression equation	y=928.92x + 289.26

A variance analysis was carried out and its results showed that concentration of sodium bicarbonate was of notable effect compared to other factors on maximum product formation of DNP-ASP. From table-1 it could be seen that DNP-ASP in extractions of ethyl acetate would not be detected from treatment 1 to treatment 4 when equal volume of water was added into the reaction system instead of sodium bicarbonate solution. In addition, the maximum product formation was increased by adding higher concentration of sodium bicarbonate solution. And it was found that treatment 11 i.e., concentrations of 0.2 mol/L of sodium bicarbonate with reaction time of 40 minutes and reaction temperature of 60°C was optimum for maximum product formation. Several extraction solvents like ethyl acetate, chloroform and hexane were tried, but ethyl acetate was found to be most suitable for better results.

It was found that DNFB and DNP-ASP both were extracted into the ethyl acetate. If we apply spectrophotometric method for the determination of aspartame then there was a possibility of interference from DNFB. But chromatographic method (HPLC) was found to be suitable where DNFB and DNP-ASP were well separated on chromatographic column. And the DNP-ASP was determined in aspartame samples without any interference from DNFB.

Chromatographic measurement

The present HPLC method gives the well separated peaks of DNP-ASP and DNFB with a short analysis time of only 7 minutes as shown in fig 3. Aspartame derivative was eluted at 4.49 minute. All the measurements were performed at wavelength of 332 nm which was based upon the UV-spectral characteristics of the DNP-ASP derivative obtained invitro (Fig 4). All the measurements were performed at ambient temperature which gave efficient resolution with out any tailing. The mobile phase was also optimized by varying mobile phase compositions and flow rate. It was found that mobile phase (hexane: ethyl acetate, 60:40 V / V, pH was adjusted to 4.5 using 1% acetic acid) with a flow rate of 1ml/min was found to be suitable for elution of aspartame with the well resolved peaks. The derivative was found to be stable up to 18 hours in ethyl acetate extract at room temperature.

The limit of detection (LOD), limit of quantification (LOQ), and linearity range (Fig 5) and coefficient correlation were found to be 1 ng, 4 ng, 20-180 ng and 0.9998 respectively (Table-2). The recovery of this method was found to be better than 97% (Table-3), with the standard deviation (+ SD) of this method was found to be less than 2.8 (< 2.8). The method remained unaffected, by small but deliberate variations, in the LC flow rate (±10%) and in the mobile phase ratio (±10%) in mobile phase. Intraday and interday studies also shows good reproducibility in respect of recovery (Table-4&5), with the precision value for intraday and interday was found to be less than 0.05 (<0.05) and 0.09 (<0.09) respectively. And the proposed method was also compared with the official method in terms of recovery in aspartame samples which was also in good agreement (table-6) with official method with a standard deviation less than 0.6 (<0.6).
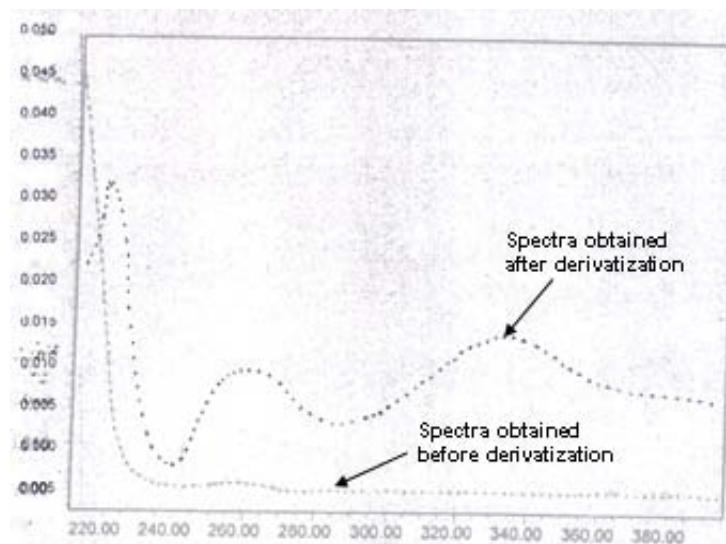

Fig. 4: Showing the spectra obtained for aspartame before and after derivatization

Tablet 4: Intraday repeatability data and precision value

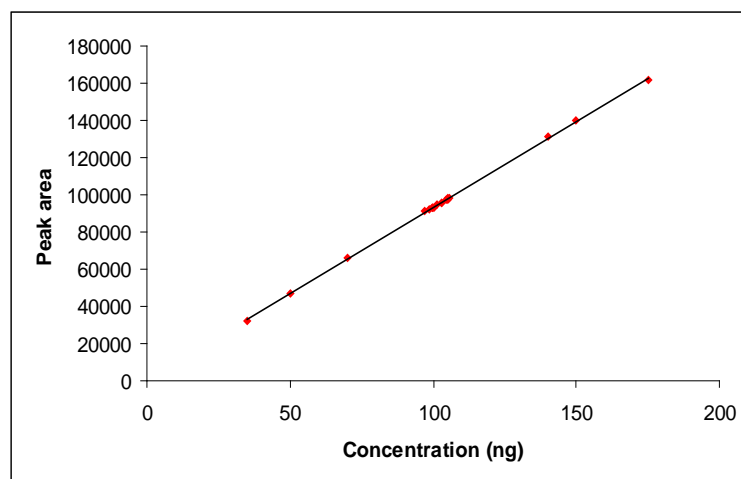| S. No	Sample	Amount found (mg)	Mean + S.D. (n=6)
1.	Spiked with 2.5 mg of aspartame	2.45, 2.39, 2.33, 2.41, 2.40, 2.46	2.40 + 0.05
2.	Spiked with 5 mg of aspartame	4.86, 4.91, 4.81, 4.79, 4.86, 4.81	4.84 + 0.04
3.	Spiked with 7.5 mg of aspartame	7.36, 7.39, 7.31, 7.41, 7.31, 7.27	7.34 + 0.05

Fig. 5: Showing the calibration obtained for aspartame with the linear regression equation of y=928.92x + 289.26 with regression value (R²) of 0.9998

Table 3: Showing the precision, accuracy/recovery values in spiked and tabletop sweeteners samples

S. No	Sample	Amount found (mg)	+ S.D.	% Recovery + S.D.
1.	Spiked with 2.5 mg of aspartame	2.45	0.03	98 + 2
2.	Spiked with 5 mg of aspartame	4.86	0.05	97.2 + 2.8
3.	Spiked with 7.5 mg of aspartame	7.38	0.02	98.4 + 1.6
4.	Tablet-1 (label claimed 10 mg)	9.83	0.05	98.3 + 1.7
5.	Tablet-2 (label claimed 10 mg)	9.87	0.01	98.7 + 1.3
6.	Tablet-3 (label claimed 10 mg)	9.37	0.07	93.7 + 6.3
7.	Tablet-4 (label claimed 10 mg)	10.46	0.12	104.6 + 4.6
8.	Tablet-5 (label claimed 10 mg)	10.22	0.11	102.2 + 2.2
9.	Tablet-6 (label claimed 10 mg)	9.31	0.13	93.1 + 6.9
10.	Tablet-7 (label claimed 10 mg)	9.71	0.09	97.1 + 2.9
11.	Tablet-8 (label claimed 10 mg)	11.05	0.11	110.5 + 10.5
12.	Tablet-9 (label claimed 10 mg)	9.97	0.23	99.7 + 0.3
13.	Tablet-10 (label claimed 10 mg)	10.34	0.18	103.4 + 3.4

Tablet 5: Interday repeatability data and precision value

S. No	Sample	Amount found (mg)	Mean + S.D. (n=3)
1.	Spiked with 2.5 mg of aspartame	2.45, 2.28, 2.35	2.36 + 0.09
2.	Spiked with 5 mg of aspartame	4.86, 4.73, 4.89	4.79 + 0.06
3.	Spiked with 7.5 mg of aspartame	7.38, 7.43, 7.29	7.4 + 0.07

Table 6: Determination of Aspartame in Tabletop sweeteners by proposed method and official method[20]

S. No	Sample	Amount found by proposed method (mg)	Amount found by official method[20] (mg)	+ S.D.
1.	Tablet-1	9.83	10.01	0.13
2.	Tablet-2	9.87	9.54	0.3
3.	Tablet-3	9.37	9.83	0.32
4.	Tablet-4	10.46	9.89	0.4
5.	Tablet-5	10.22	11.06	0.6
6.	Tablet-6	9.31	10.11	0.57
7.	Tablet-7	9.71	10.26	0.4
8.	Tablet-8	11.05	10.31	0.52
9.	Tablet-9	9.97	10.56	0.42
10.	Tablet-10	10.34	10.97	0.44

CONCLUSION

The method proposed for the determination of aspartame in tabletop sweeteners is simple, fast, sensitive, reliable and selective. Because of its high sensitivity it can be used for the detection of aspartame at trace level. As this method requires short run time, it can also be used for routine work in food, drug, forensic and environmental regulatory laboratories.

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