

HPLC DETERMINATION OF DIMETHYL-4,4'-DIMETHOXY-5,6,5',6' DIMETHYLENE DIOXYBIPHENYL-2,2'-DICARBOXYLATE (DDB) AND CO-ADMINISTERED DIURETICS AS AN EXTEMPORANEOUS MIXTURE IN HUMAN PLASMA

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Abstract

Background: Diuretics are co-administered with hepatoprotectant drugs for the treatment of oedema and ascitis in patients suffering from hepatic cirrhosis. This work presents an accurate and simple method for the in vivo analysis of Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (DDB), a hepatoprotectant drug, with hydrochlorothiazide (HCT) and amiloride hydrochloride (AM) diuretics in human plasma using HPLC. **Methodology/Principal Findings:** The separation was achieved on Spheri-5 RP-8 (5 μ) and Spheri-5 RP-18 (5 μ) columns (220 \times 4.6 mm i.d.) using 0.02 M disodium hydrogen phosphate, 0.12% w/v sodium laurylsulfate, 0.1% v/v triethylamine adjusted to pH 4 and acetonitrile in a ratio 65:35 v/v as mobile phase. The separation was carried out at ambient temperature with a flow rate of 1.0 mL min⁻¹. Quantitation was achieved with UV detection at 278 nm based on peak area with linear calibration curves at concentration ranges 0.03–30 μ g mL⁻¹ for HCT and 0.05–30 μ g mL⁻¹ for both AM and DDB. **Conclusions:** The proposed method was successfully applied to the determination of the investigated drugs in human plasma sample obtained from a healthy male volunteer four hours after single oral dose administration of the three drugs. The proposed method was validated in terms of linearity, accuracy, precision, limits of detection and quantitation and other aspects of analytical validation.

Key words: bifendate; amiloride hydrochloride; hydrochlorothiazide; co-administered; human plasma; HPLC

1. Introduction

Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (DDB) has a scavenging effect on the active oxygen radicals^[1]. DDB was shown to protect hepatocytes against injury induced by chemical toxins such as carbon tetrachloride and D-galactosamine. DDB significantly increased the liver detoxicating ability and antagonized the mutagenicity of chemical carcinogens such as aflatoxin B and benz(a)pyrene. In addition, DDB improved apparent liver functions as evidenced by reduction in elevated levels of glutamate pyruvate transaminase (GPT), bilirubin, α -fetoprotein, as well as other symptoms of chronic hepatitis. It is currently used as a hepatoprotective agent^[2]. DDB is a non-official drug. Few methods have been reported concerning the analysis of DDB. In pharmaceutical preparations, DDB has been determined using HPLC^[3].

Diuretics (such as amiloride hydrochloride, hydrochlorothiazide and triamterene) may be given in combination with DDB for the treatment of oedema and ascitis associated with hepatic cirrhosis.

DDB has been determined as single component in plasma by several HPLC methods^[4-6]. Furthermore, several HPLC methods have been reported for the determination of hydrochlorothiazide^[7, 8] and amiloride hydrochloride^[9] in biological fluids.

Diuretics (such as amiloride hydrochloride, hydrochlorothiazide and triamterene) may be given in combination with DDB for the treatment of oedema and ascitis associated with hepatic cirrhosis.

The present work was directed towards the development of suitable HPLC method for the analysis of the hepatoprotectant drug (DDB) in combination with some diuretics (amiloride hydrochloride and hydrochlorothiazide) as co-administered drugs in human plasma of patients suffering from hepatic cirrhosis. Screening of the literature reveals that there is no reported method for the determination of DDB in such combinations. This work presents a rapid, specific, reliable and selective analytical method based on reversed phase high performance liquid chromatography with UV detection for the quantitative determination of DDB in a ternary mixture with hydrochlorothiazide (HCT) and amiloride hydrochloride (AM) in spiked human plasma.

Moreover, the method has been applied to calculate the amount of DDB, hydrochlorothiazide (HCT) and amiloride hydrochloride (AM) in human plasma sample obtained from a healthy male volunteer four hours after single oral dose administration of the three drugs.

2. Experimental

2.1. Materials and reagents

DDB was kindly supplied by EVA pharmaceuticals, Cairo, Egypt. HCT and AM were obtained from Pharco pharmaceuticals, Alexandria, Egypt. All reagents used were of analytical grade. Acetonitrile (HPLC grade, Labsan Ltd., Dublin, Ireland) was used. The water for LC was double glass distilled. Phosphate buffer solutions (0.05 M) of different pH values were tried.

2.2. Apparatus

The HPLC system comprised of Perkin-Elmer Series 200 (Pump, UV/Visible detector, Autosampler and Vacuum Degasser) with a Perkin-Elmer Chromatography interface Series 600 connected to a computer loaded with TotalChrom Workstation Perkin-Elmer Chromatography software and the computer is connected to a Hewlett-Packard LaserJet 1100 printer. Chromatographic separation was accomplished on Spheri-5 RP-8 (5 μ) and Spheri-5 RP-18 (5 μ) columns (220 \times 4.6 mm i.d., Perkin-Elmer BrownLee columns). Centrifugation was performed using Sigma laboratory centrifuge 3K-30, Germany.

2.3. Chromatographic conditions

Separation of DDB, HCT and AM was performed using mobile phase consisting of 0.02 M disodium hydrogen phosphate, 0.12% w/v (0.12 gm per total 100 mL of mobile phase) sodium laurylsulfate, 0.1% v/v (0.1ml per total 100ml of mobile phase) triethylamine adjusted to pH 4 (with 0.2M orthophosphoric acid) and acetonitrile in a ratio 65:35 v/v. The mobile phase was filtered and degassed by passing through a 0.45 μ m pore size membrane filter (Millipore, Milford, MA, USA) prior to use. The samples were also filtered using 0.45-mm disposable filters. The flow rate was 1.5 mL min⁻¹ and the injection volume was 50 μ L. All determinations were performed at ambient temperature with a detection wavelength of 278 nm.

2.4. Standard Solutions and Calibration Graphs

Stock solutions of both DDB and HCT 100 μ g mL⁻¹ in acetonitrile and AM (100 μ g mL⁻¹) in water were prepared. The frozen plasma was thawed to be used at room temperature. Separate aliquots of 2 mL plasma were transferred into a set of centrifuge tubes. The plasma sample in each tube was spiked with suitable amounts of standard HCT, AM or DDB solutions to obtain final concentrations within the ranges stated in table 2. Protein precipitation was performed by adding 2 mL acetonitrile to each sample followed by centrifugation for 15 minutes at 4000 g. The collected centrifugate of each sample was evaporated under nitrogen at room temperature then the residue was re-dissolved in 200 μ L mobile phase. These final solutions were assayed under the chromatographic conditions mentioned above. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph for each compound.

2.5. Analysis of human plasma samples

2.5.1. Spiked human plasma

Separate aliquots of 2 ml plasma were transferred into a set of centrifuge tubes. The plasma sample in each tube was spiked with suitable amount of standard HCT, AM and DDB solutions as presented in table 4 and the procedure was completed as mentioned under construction of calibration graphs.

2.5.2. Human plasma samples

Human plasma sample was obtained from healthy male donor 4 hrs following a single oral dose of 10 pillules of DDB[®] (equivalent to 15 mg DDB) and one Moduretic[®] tablet (containing 5 mg AM and 50 mg HCT). Two ml aliquot of the sample was transferred into a centrifugal tube, 2 ml acetonitrile were added for protein precipitation and the procedure was completed as mentioned under construction of calibration graphs.

3. Results and discussion

The absorption spectrum of DDB in methanol exhibited single maxima at 278 nm while that of HCT in methanol exhibited three maxima at 226, 273 and 317 nm. The absorption spectrum of AM in water shows 2 maxima at 285 and 363 nm. Detection at 278 nm provided greater sensitivity for analysis of the three drugs.

The previously described experimental conditions allowed the simultaneous determination of HCT, AM and DDB with retention times of 1.81, 3.88 and 9.65 min., respectively (Fig. 1). The calculated chromatographic characteristics are listed in table 1. The three compounds are well resolved (good resolution and selectivity values) with reasonable run time (suitable capacity factors). In addition, the high column efficiency was indicated from the large number of theoretical plates.

3.1. Optimization of chromatographic conditions

Different mobile phases were used in order to achieve the best separation and resolution of the eluted peaks. The effect of acetonitrile content, concentration of sodium lauryl sulfate (SLS), concentration of triethylamine (TEA) as well as the effect of pH of the mobile phase on the capacity factor (K') values and number of theoretical plates (N) were studied. The best resolution could be achieved upon using a mobile phase consisting of 0.02M disodium hydrogen phosphate, 0.12% w/v SLS, 0.1% v/v TEA adjusted to pH 4 and acetonitrile in a ratio of 65 : 35 v/v.

3.1.1. Effect of acetonitrile content in the mobile phase

The mixture of standards was injected with mobile phases containing different proportions of acetonitrile. Figure 2 shows the retention times obtained for the three compounds as a function of acetonitrile content in the mobile phase. 35% acetonitrile was chosen to provide optimum separation with the most symmetric and well defined peaks. At lower acetonitrile concentrations, separation occurred but with excess tailing and increased retention times for DDB. Increasing acetonitrile concentrations led to overlap of HCT peak with the solvent front due to the decrease in the retention time.

3.1.2. Effect of sodium lauryl sulfate (SLS) concentration

SLS was essential to improve the sharpness and symmetry of HCT and AM peaks. Figure 3 shows the effect of SLS concentrations on the retention time of the two drugs. It causes a slight increase in the retention times of HCT and AM while nearly no change in the retention time of DDB. A concentration of 0.12 % w/v SLS in the mobile phase was found optimum and produced maximum sharpness and symmetry of HCT peak.

3.1.3. Effect of triethylamine (TEA) concentration

TEA was essential to improve the sharpness and symmetry of HCT and AM peaks. As TEA concentration increases, the retention time of the three drugs was slightly decreased (Fig. 4). An intermediate concentration of 0.1% v/v was found to be optimum to obtain maximum sharpness and symmetry of the peaks.

3.1.4. Effect of pH

The influence of pH of the aqueous phase was studied by using 0.02M solutions of disodium hydrogen phosphate containing 0.12% w/v SLS and 0.1% v/v TEA at various pH values between 3.0 - 7.0 (adjusted with orthophosphoric acid or sodium hydroxide).

These solutions were used with 35% acetonitrile as the mobile phase for a mixture of the three drugs. As the pH increased, the retention times of both HCT and DDB were slightly decreased while that of AM was slightly increased (Fig. 5). pH 4 was chosen as intermediate pH providing maximum separation of HCT peak from the solvent front.

3.2. Assay of human plasma samples

Peak plasma concentration of HCT is (0.18 - 0.43 $\mu\text{g mL}^{-1}$) with a mean of 0.26 $\mu\text{g.ml}^{-1}$ attained in 2-4 hrs following oral dose of 50 mg given to 8 subjects. A steady state plasma concentration of 0.05 - 0.16 $\mu\text{g mL}^{-1}$ (mean of 0.1) attained after daily oral doses of 75 mg to 8 subjects^[10]. For AM, peak plasma concentrations of about 0.05 $\mu\text{g mL}^{-1}$ were attained in 4 hrs after a single oral dose of 20 mg to 6 subjects^[10]. Meanwhile, plasma concentration profile of DDB was found to reach its peak (25.2 - 47.4 ng mL^{-1}) at $2.21 \pm 1.16 \text{ hr}^{[11]}$ following an oral administration to adult volunteers.

The prime focus of the study was to develop, validate and apply an analytical HPLC method intended to measure the concentrations of the three co-administered drugs at the therapeutic plasma concentration range after single oral dose administration of the three drugs.

The proposed method was applied for the determination of HCT, AM and DDB in spiked human plasma. In order to remove matrix interference that might overlap with the compounds of interest, samples were subjected to a clean-up procedure. In this respect, acetonitrile was used for protein precipitation.

Furthermore, plasma sample obtained from a healthy male volunteer 4 hrs following a single oral dose of 10 pillules of DDB[®] (equivalent to 15 mg DDB) and one Moduretic[®] tablet (containing 5 mg AM and 50 mg HCT) was analyzed using the proposed HPLC method. HCT, AM and DDB concentrations calculated in the plasma of the donor, were found to be 123, 21.4 and 37.3 ng mL^{-1} , respectively. The obtained plasma concentrations were in good agreement with the reported values for HCT and DDB while the plasma concentration for AM was slightly higher than that mentioned in the literature. This may be attributed to individual variations in the nature, rate of absorption and metabolic rate.

4. Validation

4.1. Linearity

The quantitative determination of HCT, AM and DDB was carried out based on the linear dependence of the peak area on the concentration ($\mu\text{g mL}^{-1}$). Linearity was evaluated by determining five standard working solutions of each drug within the linearity ranges stated in table 2 in triplicates. Peak area and concentration was subjected to least squares linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the working concentration ranges of the three drugs. The linearity of calibration graphs was validated by a high value of correlation coefficient, small values of intercepts. An important statistic for indicating the random error in the estimated values of y is the standard error of the estimate, or the standard deviation about regression, or the standard deviation of the residuals, $S_{y/x}$. The smaller the

standard error of the estimate, the closer will be the points to the straight line. Also, the importance of $S_{y/x}$ originates from being used to calculate S_a and S_b , the standard deviation of the intercept (a) and the slope (b). In addition, the standard deviation of slope % values (S_b %) were less than 2%. For more confirmation, the Student's t-test was performed to determine whether the experimental intercept (a) of the above-mentioned regression lines was not significantly different from the null hypothesis. The calculated values of $t (a/S_a)$ do not exceed the 95% criterion of $t = 2.31$ for 5 samples. So the intercepts are not significantly different from zero in the proposed methods. Thus, the hypothesis that (a) is of negligible value is confirmed. Linearity results are depicted in table 2 for the proposed HPLC method.

4.2. Detection and quantitation limits

Limit of detection (LOD) was defined as the concentration which had a signal to noise ratio of 3:1. For the limit of quantitation LOQ, the signal to noise ratio considered was 10:1^[12]. The LOD and LOQ values for the drug were calculated and presented in table 2.

4.3. Precision and accuracy

In order to assess the precision, as percentage relative standard deviation (RSD %) and the accuracy, as percentage relative error (Er %) of the proposed HPLC method, three replicate determinations were carried out on HCT/AM/DDB synthetic mixtures of different proportions. The data shown in table 3 indicate good precision and accuracy of the proposed method.

The within-day precision and accuracy for the determination of the three drugs in plasma were conducted using three replicate determinations for each concentration of the three drugs. The between-day precision and accuracy were similarly evaluated on several days over two weeks and no more than one assay per day for each concentration. The data presented in table 4 indicate that the within-day and between-day coefficients of variation (RSD %) and the percentage relative error (Er %) for HCT, AM and DDB are generally lower than 3%.

4.4. Selectivity

The selectivity of the method was assessed by carrying out the chromatographic procedure on blank plasma samples (after being cleaned-up) and no interfering peaks were detected at the retention times of the analytes, it was then concluded that no endogenous substances from the plasma interfered with the assay.

The stability of the column was not affected by the injection of plasma samples (after deproteinization). The resolution as well as the retention times of the drugs peaks remained constant over two weeks of work.

Table 1: Chromatographic characteristics of HCT, AM and DDB.

Compound	Retention Time, min.	Number of theoretical Plates, N	Capacity Factor, K'	Selectivity, α	Resolution, R
HCT	1.81	2318	0.65	2.14	6.24
AM	3.88	1594	2.53	2.49	9.66
DDB	9.65	6031	7.77		

Both the selectivity and resolution were calculated for each two successive peaks.

Table 2: Regression and statistical parameters for the determination of a mixture of HCT, AM and DDB by the proposed HPLC method.

Parameter	HCT	AM	DDB
Linearity range ($\mu\text{g}\cdot\text{ml}^{-1}$)	0.03-30	0.05-30	0.05-30
Regression equation			
Intercept (a)	-2.97×10^4	3.78×10^4	4.74×10^3
Slope (b)	9.86×10^4	1.57×10^5	9.93×10^4
Correlation coefficient (r)	0.9998	0.9999	0.9999
S_a	1.02×10^5	1.03×10^5	3.03×10^3
S_b	1.22×10^3	1.27×10^3	67.55
$S_{y/x}$	1.54×10^3	1.75×10^3	5.93×10^3
LOD ($\text{ng}\cdot\text{ml}^{-1}$)	8	14	15
LOQ ($\text{ng}\cdot\text{ml}^{-1}$)	27	45	48

S_a : Standard deviation of the intercept, S_b : Standard deviation of the slope, $S_{y/x}$: Standard deviation of residuals, LOD : Limit of detection, LOQ : Limit of quantitation.

Table 3: Evaluation of the precision and accuracy for the determination of HCT, AM and DDB in synthetic mixtures by the proposed HPLC method.

Nominal value ($\mu\text{g}\cdot\text{ml}^{-1}$)			Mean % recovery \pm SD			RSD (%)			Er (%)		
HCT	AM	DDB	HCT	AM	DDB	HCT	AM	DDB	HCT	AM	DDB
0.03	0.1	0.1	100.25 \pm 1.262	100.06 \pm 1.517	100.20 \pm 1.214	1.259	1.516	1.212	0.25	0.06	0.20
2	0.5	0.45	100.08 \pm 0.233	100.08 \pm 0.226	99.66 \pm 0.600	0.233	0.226	0.602	0.08	0.08	-0.34
1	8	2	99.94 \pm 0.596	99.05 \pm 0.548	99.84 \pm 0.647	0.596	0.553	0.648	-0.06	-0.95	-0.16
8	2	8	100.00 \pm 0.348	99.47 \pm 0.458	99.92 \pm 0.492	0.348	0.460	0.492	0.00	-0.53	-0.08
15	10	10	100.19 \pm 0.490	99.19 \pm 0.409	100.40 \pm 0.901	0.489	0.412	0.897	0.19	-0.81	0.40

Mean \pm standard deviation of three determinations.

Table 4: Assay results for the determination of HCT, AM and DDB spiked to human plasma by the proposed HPLC method.

Spiked conc. ($\mu\text{g.ml}^{-1}$)			Measured conc. ($\mu\text{g.ml}^{-1}$)			Mean % recovery \pm SD			RSD (%)			Er (%)		
HCT	AM	DDB	HCT	AM	DDB	HCT	AM	DDB	HCT	AM	DDB	HCT	AM	DDB
(a) Within-day precision and accuracy														
0.1	0.015	0.03	1	0.15	0.3	97.90 \pm 1.809	98.34 \pm 1.532	97.31 \pm 1.691	1.848	1.558	1.670	-2.10	-3.66	-2.69
0.2	0.05	0.045	2	0.5	0.45	98.05 \pm 1.431	101.04 \pm 1.325	97.91 \pm 1.024	1.416	1.311	1.025	-1.95	-2.96	-2.09
0.5	0.5	0.1	5	5	1	98.11 \pm 1.595	101.38 \pm 0.678	96.24 \pm 1.353	1.593	0.669	1.363	-1.89	-1.62	-3.76
1	1	1	10	10	10	97.42 \pm 0.933	102.71 \pm 0.421	98.51 \pm 0.876	0.938	0.410	0.863	-2.58	-2.29	-1.49
2	2	3	20	20	30	98.97 \pm 0.979	99.72 \pm 1.123	97.83 \pm 0.997	0.989	1.126	0.989	-1.03	-2.28	-2.17
(b) Between-day precision and accuracy														
0.1	0.015	0.03	1	0.15	0.3	96.26 \pm 2.771	101.71 \pm 1.935	97.51 \pm 3.035	2.820	1.902	2.806	-3.74	-4.29	-2.49
0.2	0.05	0.045	2	0.5	0.45	97.34 \pm 1.855	100.39 \pm 2.126	98.71 \pm 2.133	1.867	2.118	2.139	-2.66	-1.61	-1.29
0.5	0.5	0.1	5	5	1	97.61 \pm 2.128	101.53 \pm 1.125	98.13 \pm 1.745	2.094	1.108	1.778	-2.39	-2.47	-1.87
1	1	1	10	10	10	98.33 \pm 1.908	99.09 \pm 0.981	96.35 \pm 1.671	1.902	0.990	1.682	-1.67	-2.91	-3.65
2	2	3	20	20	30	98.67 \pm 1.865	98.97 \pm 1.133	96.35 \pm 2.041	1.853	1.145	2.014	-1.33	-1.03	-3.65

Mean \pm standard deviation of three determinations.

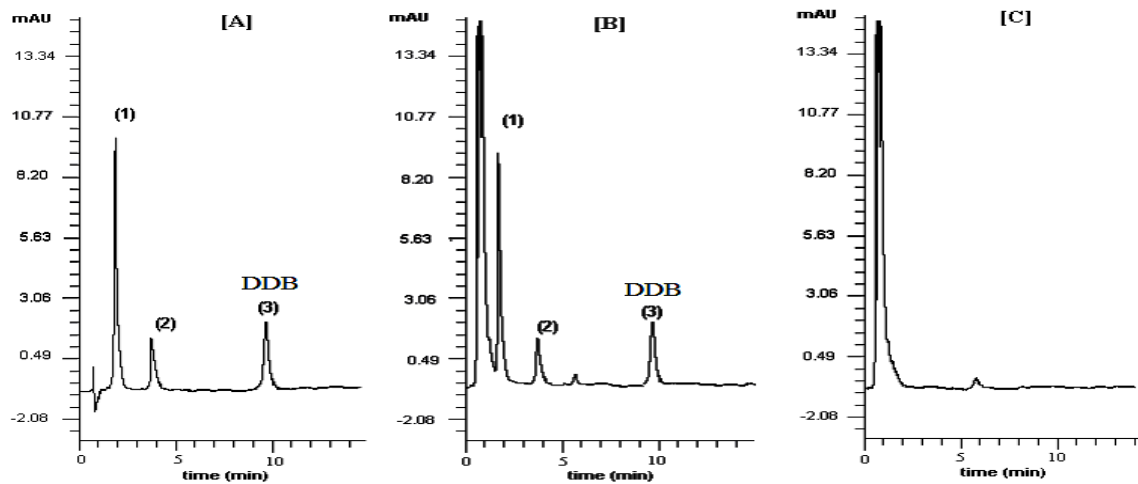


Fig. 1. Typical chromatogram of 50 μ l injection of [A] standard mixture of (1) 2 μ g. ml^{-1} HCT, (2) 0.05 μ g. ml^{-1} AM and (3) 0.45 μ g. ml^{-1} DDB, [B] Plasma spiked to give the same concentration as in standard mixture and [C] drug-free plasma.

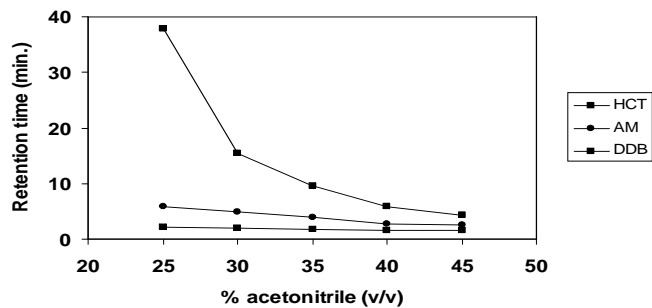


Fig. 2. Variation in the retention times of HCT, AM and DDB as a function of the percentage of acetonitrile in the mobile phase.

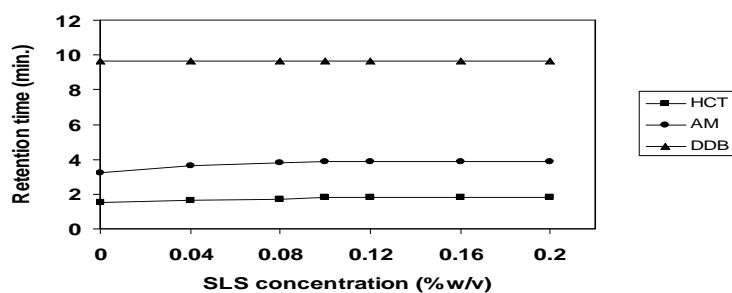


Fig. 3. Variation in the retention times of HCT, AM and DDB as a function of the concentration of a SLS (%w/v) in the mobile phase.

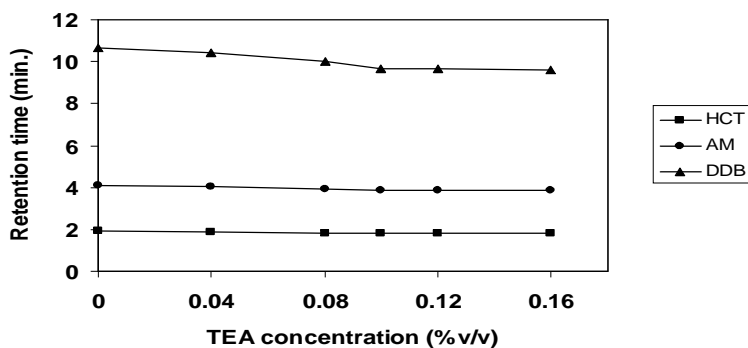


Fig. 4. Variation in the retention times of HCT, AM and DDB as a function of the concentration of a TEA (%v/v) in the mobile phase.

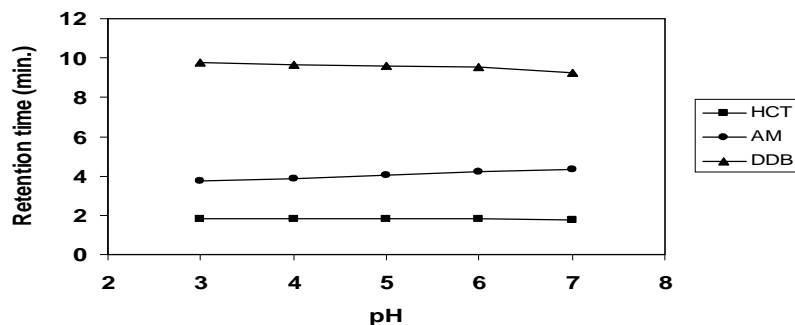


Fig. 5. Variation in the retention times of the HCT, AM and DDB as a function of pH of the aqueous phase.

5. Conclusion

A validated HPLC method has been developed for the determination of HCT, AM and DDB co-administered drugs in human plasma. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including linearity, accuracy, precision and selectivity. Its chromatographic run time of 11 min allows the analysis of a large number of samples in a short period of time. In addition, there are no previously reported HPLC methods for the simultaneous determination of DDB in such combination making the proposed method of great interest to be adopted for biopharmaceutical studies of co-administered HCT, AM and DDB in human plasma of patients suffering from hepatic disorders associated with edema.

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