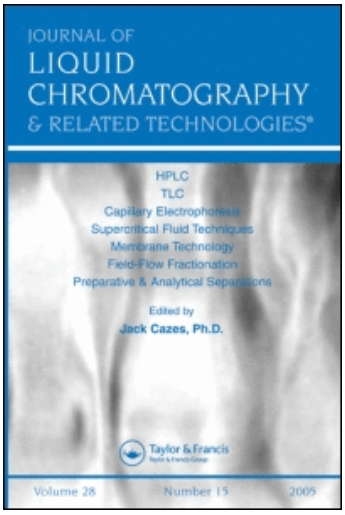

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Chromatographic and Densitometric Analysis of Hydrochlorothiazide, Walsartan, Kandesartan, and Enalapril in Selected Complex Hypotensive Drugs

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Abstract: A new chromatographic and densitometric method was developed for identification and determination of hydrochlorthiazide, walsartan, kandesartan, and enelapril present together in various complex drugs used in hypertension therapy. The TLC F_{254} plates were used as the stationary phase and two mobile phases were used of the following composition: ethyl acetate-tetrahydrufuran-acetic acid (8:2:0.5 v/v) (I) to determine kandesartan and walsartan present together with hydrochlorthiazide, and butane-1-ol-glacial acetic acid-water (12:3:5 v/v/v) (II) to determine enalapril and hydrochlorthiazide. The densitometric measurements were made at $\lambda = 252$ nm for walsartan and kandesartan and at $\lambda = 274$ nm and $\lambda = 208$ nm, for enalapril and hydrochlorthiazide, respectively. The method was specific to analyte constituents and was of high sensitivity; LOD ranged from 0.036 μg to 0.639 μg, LOQ from 0.210μg to 1.937 μg, recovery varied from 93.96% to 101.74%, the range of linearity was 0.078 μg to 6.150 μg. The results obtained for the drugs under examination was of similar precision, RSD ranged from 0.41 to 1.14.

Keywords: Densitometry, Drug analysis, Enalapril, Hydrochlorthiazide, Kandesartan, TLC, Walsartan

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INTRODUCTION

The complex hypotensive drugs play an important role in therapy of circulatory diseases and are commonly used in medical treatment. They reduce arterial blood pressure by increasing diuresis and decreasing vascular resistance.

The pharmaceutical preparations under consideration include hydrochlorthiazide of diuretic action that is present together with angiotensive receptor antagonists (walsartan and kandesartan) and enalapril as angiotensin converting enzyme inhibitor (ACE inhibitor).

As follows from the review of available literature, to determine individual constituents of hypotensive drugs in pharmaceutical preparations, as well in biological materials, the following methods are used: spectrophotometry[1–3] and derivative spectrophotometry.[4–9] In addition, liquid chromatography,[10–12] high performance liquid chromatography,[13–19] gas chromatography combined with mass spectrometry,[20] thin layer chromatography,[21,22] and capillary electrophoresis[23,24] are widely used. Also, ELIZA enzymatic method,[25] immunological method,[26] spectrofluorometry,[27] polarography,[1] AAS,[2] and NMR[28] were used for analytical purposes.

The aim of this paper is to develop a chromatographic and densitometric method by using the UV detection for identification and simultaneous quantitative determination of hydrochlorthiazide, walsartan, kandesartan, and enelapril which are present together in various complex drugs. The research studies we undertook were justified because of a lack of similar reports in the literature.

EXPERIMENTAL

Apparatus

(a) TLC- Scanner 3 densitometer with the Cats 4 software, fitted with Linomat IV, manufactured by Camag (Munthez, Switzerland);
(b) HPTLC aluminium plates, coated with silica gel, 20 × 20 cm sheets, (Merck Gemany), cut to 10 cm × 10 cm for analytical purposes;
(c) Chromatographic chamber 18 cm × 9 cm × 18 cm (Sigma Aldrich);
(d) Analytical balance, model WPA 60/C, accuracy: 0.1 mg (Radwag Poland).

Standards and Preparations

The following standards that met the European Pharmacopoeia requirements were used:

- hydrochlorthiazide (LGC Promochem, catalog code: Warsaw, Poland);
- walsartan (LGC Promochem, Warszawa, Poland);
- kandesartan (LGC Promochem, Warszawa, Poland);
- enlapril (LGC Promochem, Warszawa, Poland);
- Co-Diovan, tablets manufactured by Novartis Pharma, of the following composition: 80 mg of walsartan and 12.5 mg of hydrochlorthiazide per tablet;
- Blopress Plus, tablets manufactured by Takeda Pharma, of the following composition: 16 mg of kandesartan cilexetil and 12.5 mg of hydrochlorthiazide per tablet;
- Enap HL, tablets manufactured by KRKA, of the following composition: 10 mg enelapril maleate and 12.5 mg of hydrochlorthiazide per tablets.

Standard Solutions

The standard solutions were prepared by dissolving weighed amounts of the standards in methanol to obtain solutions of the following concentrations:

- Hydrochlorthiazide: 0.38 mg/mL, 0.34 mg/mL, 0.32 mg/mL, 0.16 mg/mL, 0.08 mg/mL, 0.04 mg/mL, 0.02 mg/mL;
- Walsartan: 3.31 mg/mL, 1.23 mg/mL, 0.615 mg/mL, 0.48 mg/mL, 0.3076 mg/mL, 0.153 mg/mL, 0.077 mg/mL;
- Kandesartan: 0.723 mg/mL, 0.58 mg/mL, 0.362 mg/mL, 0.181 mg/mL, 0.090 mg/mL, 0.045 mg/mL;
- Enalapril: 1.0 mg/mL, 0.50 mg/mL, 0.40 mg/mL 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL.

Tested Solutions

Twenty tablets were taken from each batch of the drugs under investigation for testing purposes and the mean weight was calculated. The tablets were then powdered in a mortar. The weighed samples were made from powdered tablets according to the mean tablet weight. After adding 10 mL of methanol, the individual samples were shaken for 15 minutes, afterwards centrifuged, and the supernatant was taken. For direct determinations, the specified volumes of solutions were diluted with methanol, as required, to obtain solutions of the following concentrations: 0.625 mg/mL and 0.125 mg/mL of hydrochlorthiazide, 4.00 mg/mL of walsartan, 0.16 mg/mL of kandesartan, and 0.20 mg/mL of enelapril.

Mobile Phases

I – ethyl acetate - tetrahydrufuran - acetic acid (8:2:0.5 v/v)
II – butane-1-ol - glacial acetic acid - water (12:3:5 v/v/v)

Chromatographic Analysis

Initially, an attempt was made to establish the constituent separation conditions for preparations under investigation. For this purpose, 10 mm bands, each of 5 μL of appropriate standard and tested solutions were applied onto 10 × 10 cm chromatographic plates. Chromatograms were developed up to the height of 95 mm in a chamber saturated with the mobile phase. Among various mobile phases under examination, phase I was chosen for separating hydrochlorthiazide, kandesartan, and walsartan, and mobile phase II for separating hydrochlorthiazide and

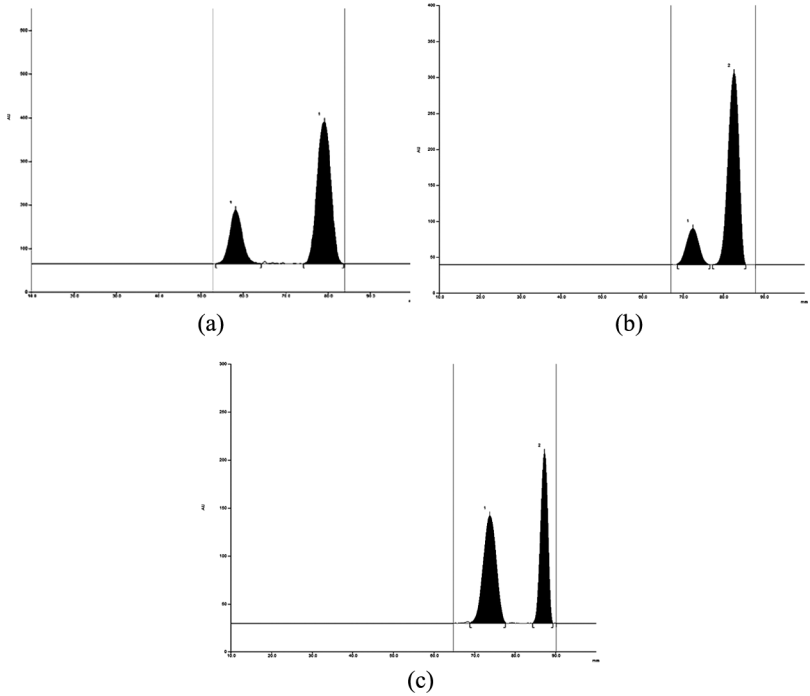

Figure 1. An example of densitograms recorded for the analyzed preparations: (a) – Enap HL, mobile phase II, enalapril (1), hydrochlorthiazide (2), (b) – Blopress Plus, mobile phase I, hydrochlorthiazide (1), kandesartan (2), (c) – Co-Diovan; mobile phase I, hydrochlorthiazide (1), od walsartan (2).

enalapril. The selected mobile phases provide a good separation of analyzed components, thus enabling their identification and quantitative determination. The values of R_F are: $R_F \sim 0.74$ for hydrochlorthiazide, $R_F \sim 0.88$ for walsartan and $R_F \sim 0.89$ for kandesartan, and $R_F \sim 0.84$ for hydrochlorthiazide and $R_F \sim 0.61$ for enalapril. There are only peaks of tested constituents on chromatograms of the tested solutions. The peak locations comply with those of the standard solutions. An example of densitograms is presented in Figure 1.

The absorption spectra, recorded directly from chromatograms, were characteristic of the constituents under investigation, with absorbance maxima at $\lambda = 252\,\text{nm}$ for walsartan and kandesartan, $\lambda = 208\,\text{nm}$ for enalapril, and $\lambda = 274\,\text{nm}$ for hydrochlorthiazide (Fig. 2).

The validation of the method was made by determining specificity, accuracy, precision, linearity, limits of detection, and limits of quantitation.

Specificity

The specificity of the method was determined by comparing the peak areas, values of R_F and absorption spectra recorded for appropriate standard, and preparation solutions of comparable constituent content.

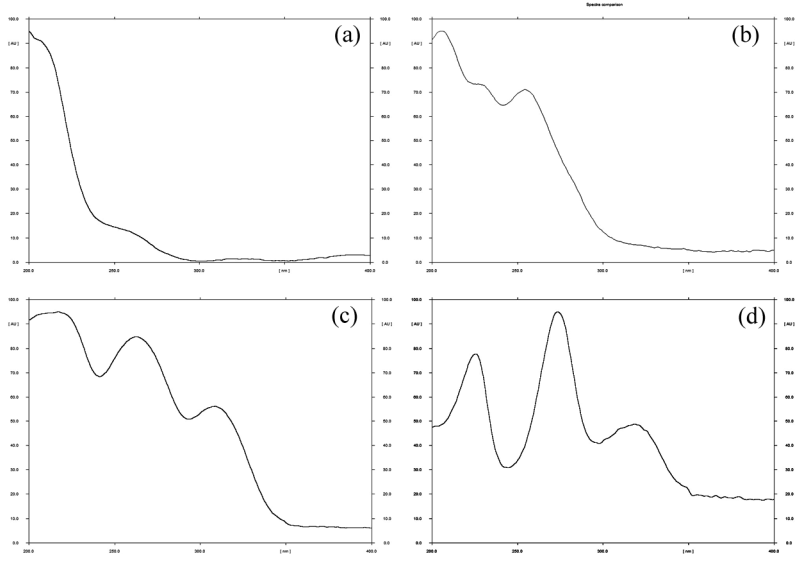

Figure 2. Spectra of enalapril (a), walsartan (b), kandesartan (c) and hydrochlorthiazide (d) recorded directly from a chromatogram.

Accuracy

The accuracy of the method was expressed by percentage recovery (%E) for constituents under investigation. To do this, an exactly known amount, ranging from 80% to 120% of the declared amount of the standard was added to the tested solutions. Determinations were carried out before adding the appropriate reference substance (c_0) and after adding it (c_x). The percentage recovery was computed in relation to the weighed amount (c_w) according to the following formula:

$$\%E = 100\% \cdot (c_x - c_0)/c_w \text{ (see Table 1).}$$

Precision

The consistency of the results of the determinations was tested on model solutions prepared by dissolving the constituents in an appropriate

Table 1. Parameters of validation for the method

Substance (mobile phase)	Enalapril (II)	Walsartan (I)	Kandesartan (I)	Hydrochlorthiazide (I)	Hydrochlorthiazide (II)
Specificity	Method specific to the examined analyte				
R_f	~0.61	~0.88	~0.89	~0.74	~0.84
LOD [μg/band]	0.172	0.639	0.245	0.069	0.036
LOQ [μg/band]	0.520	1.937	0.750	0.210	0.109
Recovery [%]	102.2	95.8	94.7	97.3	108.2
	101.5	93.4	95.1	96.7	101.0
	98.5	92.1	94.8	100.6	98.3
	105.0	97.7	93.1	96.4	99.2
	97.8	94.8	92.1	100.8	102.0
$\bar{x}$	101.00	94.76	93.96	98.42	101.74
S_x	2.923	2.159	1.299	2.0969	3.893
$t_{95\%}$	±3.630	±2.681	±1.613	±2.6036	±4.834
RSD	2.89	2.28	1.38	2.13	3.83
Precision[mm^2]	4219.2	3578.0	3594.6	7529.8	5155.6
	4174.6	3671.0	3615.2	7464.8	5165.8
	4204.2	3589.2	3628.4	7621.3	5032.5
	4185.4	3658.1	3650.0	7501.2	5090.4
	4191.2	3638.4	3669.0	7517.8	5198.1
$\bar{x}$	4194.9	3626.9	3631.4	7471.8	5128.5
S_x	17.27	41.42	29.09	63.36	29.70
$t_{95\%}$	±21.44	±51.43	±36.12	±78.67	±82.45
RSD	0.41	1.14	0.80	0.84	0.58
Regression equation	$p = 2144.2 \cdot m$	$p = 1826.0 \cdot m$	$p = 3351.0 \cdot m$	$p = 3105.2 \cdot m$	$p = 10170.0 \cdot m$
Correlation coefficient	+141.4 $r = 0.99903$	+1584.8 $r = 0.99748$	+561.4 $r = 0.99627$	+286.3 $r = 0.99956$	+294.8 $r = 0.99933$

R_f – retention coefficient; $\bar{x}$ – mean; S_x – standard deviation, $t_{95\%}$ – confidence interval for probability of 95%; RSD – relative standard deviation; p – peak area [mm^2]; c – concentration [mg/ml]; r – correlation coefficient

solvent. For each solution of the following composition: 0.58 mg of kandesartan, 0.48 mg of walsartan, and 0.34 mg of hydrochlorthiazide in 1 mL and 0.40 mg of enalapril and 0.20 mg of hydrochlorthiazide in 1 mL, 5 determinations were made (see Table 1).

Linearity

The linearity was found through the relationship between peak areas (p[mm^2]) and masses m[μg/spot]. The measure of linearity for constituents under investigation was expressed with equations of a straight line of the following correlation coefficients (r):

- hydrochlorthiazide: $p = 3105.2 \cdot m + 286.3$, $r = 0.99956$, system I
- hydrochlorthiazide: $p = 10170.0 \cdot m + 294.8$, $r = 0.99933$, system II
- kandesartan: $p = 3351.0 \cdot m + 561.4$, $r = 0.99627$
- walsartan: $p = 1826.8 \cdot m + 1584.8$, $r = 0.99211$
- enalapril: $p = 2144.2 \cdot m + 141.4$, $r = 0.99903$.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

By using both standard deviation and slope of a straight line, the limits of detection and quantitation were computed from the following equations: $LOD = 3.3 \cdot S_y/a$, $LOQ = 10 \cdot S_y/a$, where: S_y – estimation error, a – slope of a straight line (see Table 1).

As a result the analysis procedure was established.

Determination Procedure

Apply, from the bottom of the plate, 1 cm bands of 1 cm long containing 5 μL of the standard and tested solutions onto TLC plates of 10 cm × 10 cm in size. Develop chromatograms up to 9.5 cm by using the mobile phase I to separate hydrochlorthiazide along with walsartan and kandesartan and the mobile phase II to separate hydrochlorthiazide and enalapril, and then dry at room temperature. Perform densitometric recording at wavelength $\lambda = 274$ nm for hydrochlorthiazide, $\lambda = 208$ nm for enalapril and $\lambda = 252$ nm for walsartan, and kandesartan.

Compute the values of coefficients R_F for constituent identification purposes and record absorption spectra within the wavelength range from 200 nm to 400 nm. To compute the concentrations of active substances in preparations under examination, use the peak areas recorded for the tested solutions and appropriate standard solutions.

DISCUSSION

The combination of drugs belonging to different chemical groups into complex forms to achieve the specified therapeutic effect is of great importance, not only for its therapeutic value, but also from an analytical point of view. Similar physicochemical properties and the presence of auxiliary constituents in complex drugs induce new analytical procedures enabling full control of qualitative and quantitative compositions. As mentioned in this paper, the aim of the research studies undertaken was to develop a chromatographic and densitometric method enabling simultaneous identification and quantitative analysis of drug constituents. Good results were not obtained by using the same mobile phase and this is why two mobile phases were selected. Regardless of the mobile phase used, symmetrical and well developed peaks enabling identification and quantitative determination were obtained. Mobile phase I of the following composition: ethyl acetate – tetrahydrufuran – acetic acid (8:2:0.5 v/v) was chosen to identify kandesartan and walsartan present with hydrochlorthiazide, while butane-1-ol – glacial acetic acid – water (12:3:5 v/v/v) was used to dewtermine enalapril and hydrochlorthiazide.

Only peaks that originated from the constituents under examination of consistent values of R_F were present on chromatograms recorded for appropriate standard and sample solutions. The peaks were well separated and showed no interference with each other and those of the constituents of auxiliary substances present in the tablets. The newly developed method is of high sensitivity; LOD is 0.069 μg· and 0.036 μg for hydrochlorthiazide, depending on the mobile phase used, 0.639 μg for walsartan, 0.245 μg for kandesartan, and 0.172 μg for enalapril, while LOQ is: 0.210 μg, 0.109 μg, 1.937 μg, 0.750 μg, and 0.520 μg, respectively. Recovery of the determined components was: 98.42%, 101.74%, 94.76%, 93.96%, and 101.00%. The linearity range was: from 0.100 μg to 1.600 μg and from 0.078 μg to 1.250 μg for hydrochlorthiazide, from 0.385 μg to 6.150 μg for walsartan, from 0.226 μg to 1.810 μg for kandesartan and from 0.312 μg to 5.000 μg for enalapril. The results of determinations for individual constituents were of similar precision, with RSD ranging from 0.41% to 1.14%. The quantitative determinations for the constituents of the drugs under examination (Table 2), made by using the newly developed method, were of high precision and accuracy, and relative standard deviation RSD (%) ranged from 0.41 to 1.14, while the determined constituent concentrations complied with those declared by the drug manufacturers (Table 2). To summarise, one can conclude that the proposed method, in addition to the value of being a new solution, also has a practical aspect, which makes it preferable in analytical laboratories.

Table 2. The results of determinations obtained for analyzed drugs

Preparation (declared tablet content)	Hydrochlorthiazide (mg/tbl)	Kandesartan (mg/tbl)	Walsartan (mg/tbl)	Enalapril (mg/tbl)
	12.45	16.03		
Blopress Plus	12.22	16.03		
kandesartan 16 mg	12.42	16.19		
hydrochlorthiazide	12.23	16.28	–	–
12.5 mg	12.20	15.90		
	$\bar{x} = 12.30$	$\bar{x} = 16.09$		
	$S_x = 0.120$	$S_x = 0.149$		
	$t_{0,95} = \pm 0.149$	$t_{0,95} = \pm 0.185$		
	RDS = 0.98%	RSD = 0.93%		
	12.34		71.41	
Co-Diovan	12.50		74.98	
	12.62		73.79	
walsartan 80 mg	12.30	–	76.37	–
hydrochlorthiazide	12.79		75.68	
12.5 mg	$\bar{x} = 12.51$		$\bar{x} = 74.45$	
	$S_x = 0.202$		$S_x = 1.946$	
	$t_{0,95} = \pm 0.251$		$t_{0,95} = \pm 2.417$	
	RSD = 1.62%		RSD = 2.61%	
	12.70			10.10
Enap HL	12.21			10.00
	12.70			9.95
enalapril 10 mg	12.45	–	–	10.07
hydrochlorthiazide	12.45			10.16
12.5 mg	$\bar{x} = 12.55$			$\bar{x} = 10.06$
	$S_x = 0.209$			$S_x = 0.083$
	$t_{0,95} = \pm 0.260$			$t_{0,95} = \pm 0.103$
	RSD = 1.67%			RSD = 0.82%

$\bar{x}$ – mean; S_x – standard deviation, $t_{95\%}$ – confidence interval for probability of 95%; RSD – relative standard deviation

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