Simultaneous determination of hydrochlorothiazide, amlodipine besylate and olmesatan medoxomil in tablets by high performance liquid chromatography

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Abstract

Background: The combination of hydrochlorothiazide (HCT), amlodipine besylate (AML) and olmesartan medoxomil (OLM) is highly effective in treating hypertension. Currently, Vietnamese Pharmacopoeia V and other reference pharmacopoeias do not have a procedure for the simultaneous quantification of these three ingredients. Objective: To develop and validate a procedure for simultaneous determination of HCT, AML and OLM in tablets by HPLC method. **Materials and method:** Materials: tablets containing three ingredients HCT, AML and OLM. Method: Investigation of chromatographic conditions, including mobile phase compositions and elution mode, development and validation of a procedure for simultaneously quantifying HCT, AML and OLM in tablets using the HPLC method according to ICH guidelines and the Drug Registration Manual, appendix 8. **Results:** The reversed-phase high-performance liquid chromatography method was developed with chromatographic conditions: column Xterra® C18 (250 × 4.6 mm; 5 µm), PDA detector with detection wavelength 237 nm, column temperature 30 °C, flow rate 1 ml/min, sample injection volume 10 µl, gradient elution with mobile phase acetonitrile – triethylamine buffer pH 3. Results showed that these compounds were completely separated, the retention time of HCT, AML, OLM were 5.127 minutes, 9.279 minutes and 12.889 minutes, respectively. The procedure has been validated and has met the requirements about system suitability, specificity, accuracy, precision, linearity, robustness. **Conclusion:** A procedure for simultaneously quantifying these three compounds in tablets using the HPLC method has been successfully developed and validated.

Keywords: *amlodipine besylate; olmesartan medoxomil; hydrochlorothiazide; high performance liquid chromatography, simultaneous determination.*

1. INTRODUCTION

According to the World Health Organization, hypertension is a serious medical condition and can increase the risk of other diseases relating to brain, heart, kidney. This is the main cause of premature death worldwide. It is estimated that about 1.28 billion adults aged 30 - 79 years worldwide have hypertension, mainly in low- and middle-income countries [1]. The rate of hypertension continues to increase not only in the world but also in our country [2]. Therefore, treating and controlling hypertension plays an important role.

Fixed-dose combination of antihypertensive active pharmaceutical ingredients not only simplifies the treatment regimen, increases patient compliance, but also produces better antihypertensive effect and improves tolerability. A combination including angiotensin II receptor blocker, calcium channel blocker and thiazide diuretic is one of the leading combinations for patients using a combination of two kinds of hypertension drugs but not achieving effective treatment [3]. The combination of HCT, AML and OML, in addition to its blood pressure-lowering effect, also significantly reduces the incidence of edema associated with AML monotherapy and limits side effects caused by HCT [4].

Currently, combination tablets containing HCT, AML and OLM have not appeared in the Vietnamese pharmaceutical market but are only in the research and production stage. In foreign pharmaceutical markets, the drug is also quite limited.

Up to now, in Vietnam there has not been any research that simultaneously quantifies HCT, AML and OLM in tablets. In the world, there have been some research which have carried out simultaneous quantification of HCT, AML and OLM using high-performance liquid chromatography with C18

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reversed-phase column, mobile phase is a mixture of organic solvent and buffer solution, detection wavelength in range from 230 to 262 nm [5-7, 11,12].

Therefore, this study was carried out to develop a procedure for simultaneously quantifying three substances in preparations, contributing to drug quality control, helping to quantify quickly and accurately, reducing time and cost. The effort involved in quantifying each individual ingredient contributes to building in-house specifications for the product.

2. MATERIALS AND METHODS

2.1. Material and equipment

Preparation: Tablet samples contain three ingredients HCT, AML and OLM from X Pharmaceutical Joint Stock Company.

Reference substances: HCT with batch number T218148 with 99.71% content, AML with batch number T117157 with 100.6% content, origin from National Institute of Drug Quality Control. OLM batch number Y0001405 content 99.5%, origin from Sigma-Aldrich.

Solvents and chemicals: acetonitrile (HPLC grade) was from Macron Fine Chemicals, phosphoric acid (analytical grade) was from Sigma-Aldrich, triethylamine (analytical grade) was from Merck.

Equipment: Analytical equipment was Waters Alliane e2695 high-performance liquid chromatography system, Waters 2996 PDA detector, Metler Toledo S20 pH meter, Sartorius CPD225D 5-digit analytical balance, Xterra column C18 packed particle (250 mm × 4.6 mm, 5 μm). Glassware met requirements needed in analytical work.

2.2. Methods

2.2.1. Chromatographic conditions

We carried out an investigation of the following mobile phase compositions, isocratic or gradient elution:

Table 1. The chromatographic conditions were carried out

Experiment no.	Elution mode	Mobile phase	Figure
1	Isocratic	acetonitrile – phosphoric acid 0.01% at ratio 70:30 (v/v)	1.a
2	Isocratic	acetonitrile – phosphoric acid 0,01% at ratio 50:50 (v/v)	1.b
3	Isocratic	acetonitrile – phosphoric acid 0,01% at ratio 30:70 (v/v)	1.c
4	Isocratic	acetonitrile – triethylamine pH 3 at ratio 30:70 (v/v)	1.d
5	Isocratic	acetonitrile – triethylamine pH 3 at ratio 40:60 (v/v)	1.e
6	Gradient 1	Gradient program 1 (details in Table 2)	1.f
7	Gradient 2	Gradient program 2 (Details in Table 3)	1.g
8	Gradient 3	Gradient program 3 (details in Table 4)	1.h

Table 2. Gradient programs

Gradient program 1		
Time	**Acetonitrile (%)**	**Buffer triethylamine pH 3 (%)**
0	30	70
6	45	55
10	45	55
15	30	70

Gradient program 2		
Time	**Acetonitrile (%)**	**Buffer triethylamine pH 3 (%)**
0	30	70
8	45	55
12	45	55
15	30	70
Gradient program 3		
Time	**Acetonitrile (%)**	**Buffer triethylamine pH 3 (%)**
0	25	75
8	45	55
12	45	55
15	25	75

We would select chromatographic conditions in which the peaks of the quantified substances were pure, completely separated (resolution ≥ 1.5), the tailing factor was in the range of 0.8 - 1.5 and the number of apparent theoretical plates was over 2000. [10,14]

Based on investigation results, the procedure for simultaneously quantifying HCT, AML and OLM in tablet dosage forms using high-performance liquid chromatography with PDA detector was built.

2.2.2. Preparation of standard and sample solutions

- *Buffer triethylamine pH 3:* Dissolve 7.0 ml triethylamine in 1000 ml water, adjust to pH 3.0 ± 0.1 with phosphoric acid.

- *Diluent:* acetonitrile – water (75 : 25, v/v).

- *Standard mixture solution:* Accurately weigh 12.50 mg of HCT reference standard, 6.93 mg of AML reference standard (corresponding to 5.00 mg of amlodipine) and 20.00 mg of OLM reference standard into a 100 ml volumetric flask. Add about 70 ml of sample solvent, shake well and sonicate until completely dissolved, then let cool. Add sample diluent to the mark and shake well. The resulting standard mixture solution has a concentration of HCT of about 125 ppm, AML of about 69.3 ppm (corresponding to amlodipine concentration of about 50 ppm) and OLM of about 200 ppm. Filter through a 0.45 µm membrane filter.

- *Sample solution:* Weigh 20 tablets, calculate the average weight of the tablets and grind them into fine powder, mix well. Weigh an amount of powder accurately corresponding to 12.50 mg HCT, 6.93 mg AML (corresponding to 5.00 mg amlodipine) and 20.00 mg OLM into a 100 ml volumetric flask, add approximately 70 ml sample solvent, shake thoroughly and sonicate for about 15 minutes, then cool to room temperature. Next add *diluent* to the mark and shake well. Filter through filter paper, discarding the first 10 - 15 ml of filtrate. The resulting solution has a concentration of HCT of about 125 ppm, AML of about 69.3 ppm (corresponding to an amlodipine concentration of about 50 ppm) and OLM of about 200 ppm. Filter through a 0.45 µm membrane filter.

- *Chromatographic conditions*: analyze samples with column XTerra® C18 (250 × 4.6 mm; 5 µm), PDA detector is set at detection wavelength 237 nm, column temperature 30 °C, flow rate 1 ml/min, sample injection volume 10 µl, diluent is acetonitrile - water (75: 25, v/v), mobile phase includes acetonitrile - triethylamine buffer pH 3.

- *Placebo*: Accurately weigh an amount of excipient corresponding to the amount of drug powder containing 12.50 mg HCT, 6.93 mg AML (corresponding to 5.00 mg amlodipine) and 20.00 mg OLM into a 100 ml volumetric flask. Add about 70 ml of sample solvent, shake thoroughly and sonicate for about 15 minutes, then let cool. Add sample diluent to the mark and shake well. Filter through filter paper, discarding the first 10 - 15 ml of filtrate. Filter through a 0.45 µm membrane filter.

The procedure for simultaneous quantitative determination of HCT, AML and OLM by HPLC method with PDA detector was validated according to ICH guidelines issued in November 2005 [9] and according to the decision of the Director of the Drug Administration, Ministry of Health No. 07/QD-QLD dated January 11, 2013 on promulgating the Drug Registration Manual, Appendix 8 [10], including investigation of system suitability, specificity, linearity, precision, accuracy.

Accuracy

Preparation of 3 types of self-created samples by adding an amount of standard substances to placebo samples. The amount of standard substance added corresponds to 3 levels of 80%, 100% and 120%. At each concentration level, perform at least 3 independent samples.

Precision

Conducting analysis on 6 test samples, each sample once. Determination of the percentage of active ingredients compared to the amount stated on the label. The repeatability of the method is determined by the RSD value (%) of the results of quantifying the active ingredient content in the samples.

Specificity

Conducting analysis of blank sample, HCT standard solution, AML standard solution, OLM standard solution, mixed standard solution, test sample, test sample added standard solution, placebo sample, placebo sample added standard solution and other degradation samples.

Linearity

Carrying out analysis of the samples, record the chromatograms and determination of the response of the peaks in the samples. Identification of the linear regression equation and linear correlation coefficient between the concentration of standard solution in the sample and the peak response obtained on the chromatograms.

2.2.3. Forced degradation study

Thermal degradation: Take about 1 g of drug powder and put it in a petri dish, place it inside drying oven at 80 ± 2°C for 24 hours. Then, accurately weigh an amount of powder corresponding to 12.50 mg of hydrochlorothiazide, 6.93 mg of amlodipine besilate (corresponding to 5.00 mg of amlodipine) and 20.00 mg of olmesartan medoxomil into a 100 ml volumetric flask, add about 70 ml of sample solvent, shake thoroughly and sonicate for about 15 minutes, then let cool. Add sample diluent to the mark and shake well. Filter through filter paper, discarding the first 10 - 15 ml of filtrate. Filter through a 0.45 µm membrane filter.

Photo degradation: Take about 1 g of drug powder and put it in a petri dish, place the sample under sunlight for 24 hours. Then, accurately weigh an amount of powder corresponding to 12.50 mg of hydrochlorothiazide, 6.93 mg of amlodipine besilate (corresponding to 5.00 mg of amlodipine) and 20.00 mg of olmesartan medoxomil into a 100 ml volumetric flask, add about 70 ml of sample solvent, shake thoroughly and sonicate for about 15 minutes, then let cool. Add sample diluent to the mark and shake well. Filter through filter paper, discarding the first 10 - 15 ml of filtrate. Filter through a 0.45 µm membrane filter.

Oxidation degradation: Accurately weigh an amount of powder corresponding to 12.50 mg hydrochlorothiazide, 6.93 mg amlodipine besylate (corresponding to 5.00 mg amlodipine) and 20.00 mg olmesartan medoxomil into a 100 ml volumetric flask. Add about 70 ml of sample solvent, shake thoroughly and sonicate for about 15 minutes, then let cool. Add 10.0 ml of 30% H2O2 solution to the volumetric flask. Then, add sample solvent up to the mark and shake well. The resulting solution was left at room temperature for 24 hours. Filter through filter paper, discarding the first 10 - 15 ml of filtrate. Filter through a 0.45 µm membrane filter.

3. RESULTS

As shown in Figure 1.a, when chromatography was conducted with the mobile phase at ratio acetonitrile:phosphoric acid 0.01% 70:30 (v/v), three standard peaks AML, OLM, HCT appeared. However, the OLM and HCT peaks did not separate from each other, the peaks eluted very early (below 3 minutes).

At the ratio of 50:50 (Figure 1.b), the peaks appeared very early, the OLM and HCT peaks still did not separate from each other, the base of the amlodipine peak widened. At the ratio of 30:70 (Figure 1.c), the peaks of OLM and HCT were completely separated, the OLM peak is pure on the chromatogram and had a tailing factor of 1.06, and the retention time of the peaks had a clear improvement. However, the AML peak had an unusual shape (wide base, unsatisfactory tailing factor) and HCT was not pure on the chromatogram.

When the ratio of phosphoric acid was gradually increased, the retention time of the peaks improved, the peaks tended to separate from each other. However, the peak shape of amlodipine became worse. Therefore, we kept the ratio 30:70 but change the mobile phase solvent with the goal of improving the peak shape of AML and the purity of HCT on the chromatogram.

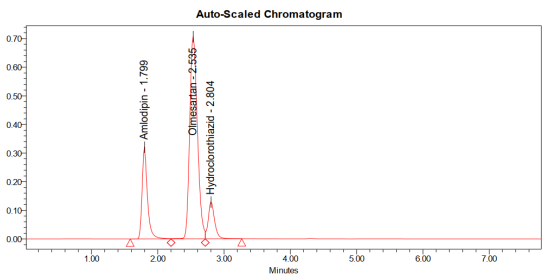
We continued conducting the investigation with the ratio of acetonitrile - triethylamine buffer pH 3 (30: 70, v/v). As shown in Figure 1.d, the peaks were completely separated from each other, all were pure on the chromatogram, the tailing factor was satisfactory, but the separation time prolonged, up to 29.054 minutes. Therefore, we increased

the proportion of organic solvent with the goal of shortening analysis time. With the organic solvent ratio 40:60, the analysis time was significantly shortened (< 10 minutes, Figure 1.e). However, the HCT peak appeared quite early (3.624 minutes), leading to poor purity on the chromatogram.

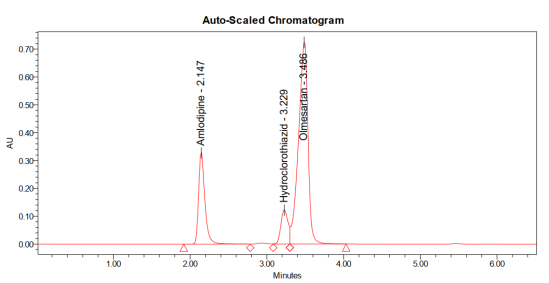
Next, acid water was replaced by triethylamine buffer, the peaks were completely separated from each other, the peak shape improved, the tailing factor was acceptable, and the number of theoretical plates increased. However, with isocratic elution mode, the retention time of the peaks were not properly distributed, and the HCT peak was not pure. Therefore, we continued to investigate in gradient elution with the goal of shortening analysis time and improving the purity of the HCT peak.

Gradient program 1 is carried out as shown in Table 2. The results showed that the peaks were completely separated, the retention time of the peaks was reasonable, the parameters of tailing factor, number of theoretical plates,... were suitable. However, the HCT peak was still not pure in the chromatogram (Figure 1.f). We changed the investigation with gradient elution program 2.

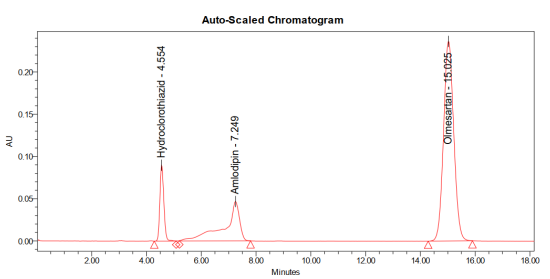
The results of the gradient program 2 (Figure 1.g) showed that the HCT peak had a retention time that did not change significantly and was not pure on the chromatogram. We continued the investigation with a new gradient elution program with the goal of increasing the retention time of the HCT peak to improve purity by reducing the proportion of organic solvents.



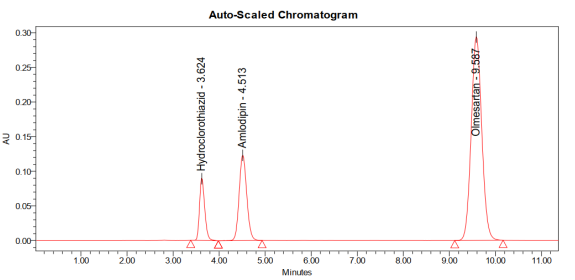
(a)



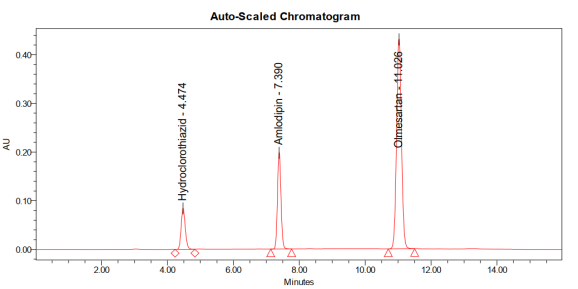
(b)



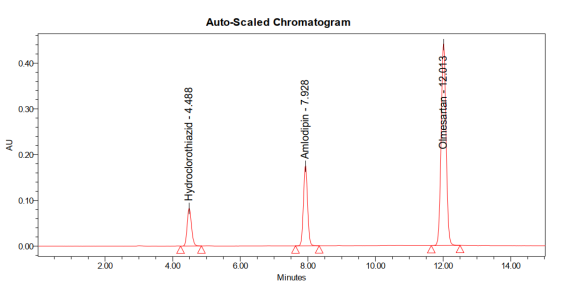
(c)



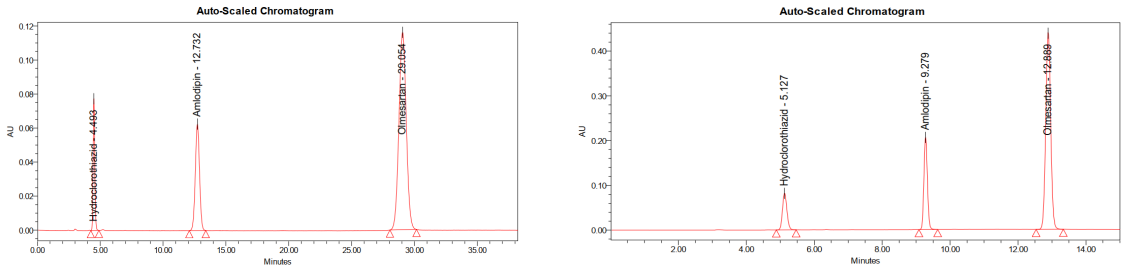
(e)



(f)



(g)

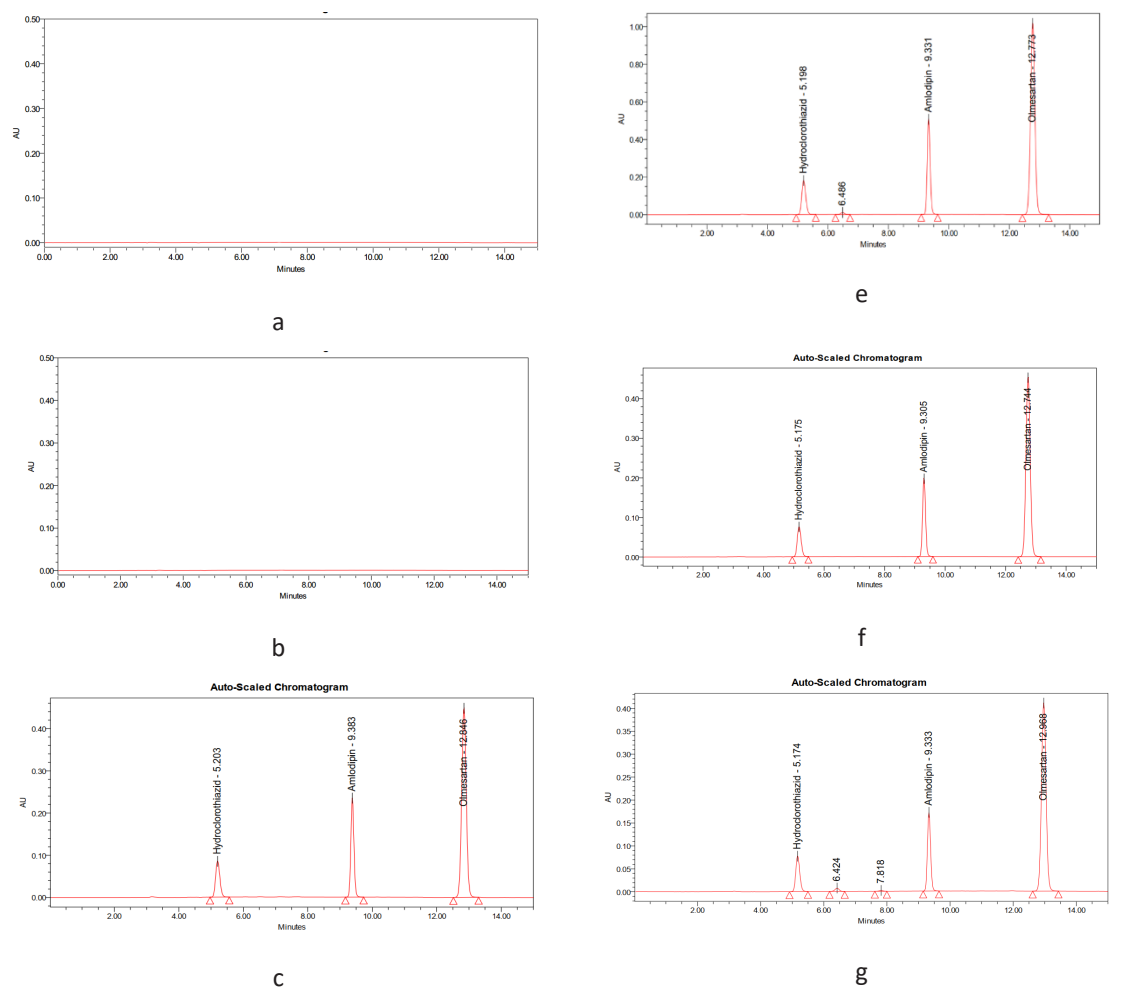


(d) (h)

Figure 1. Chromatograms obtained when analyzing the standard mixture using different mobile phase compositions and elution mode

Results with gradient program 3 (Figure 1.h) showed that the peaks were completely separated and all obtained purity on the chromatogram (purity angle < purity threshold). The retention time of the peaks was reasonably distributed. The chromatographic parameters were suitable and analysis time was short (below 15 minutes). Therefore, the mobile phase solvent system acetonitrile - triethylamine buffer pH 3 with gradient program 3 was chosen to simultaneously quantify HCT, AML and OLM.

The validation results showed that the procedure of simultaneously quantifying HCT, AML and OLM using the HPLC-PDA method obtained all the requirements according to ICH guidelines.



a e

b f

c g

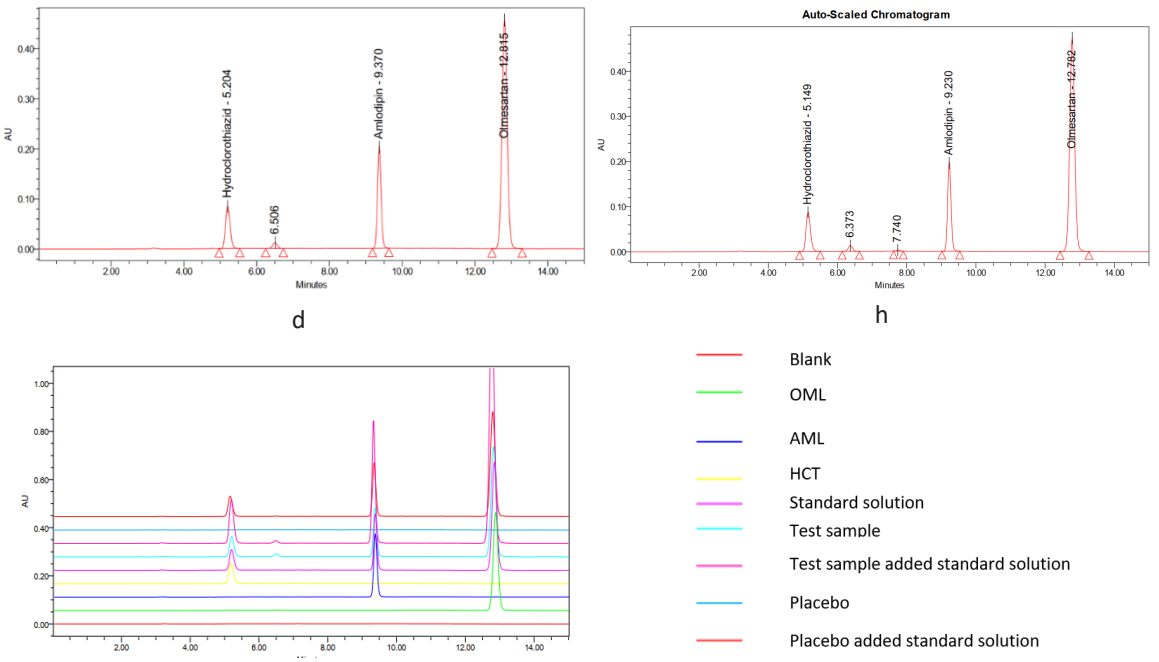


Figure 2. Chromatograms when evaluating specificity:

a. Blank b. Placebo c. Standard solution d. Test sample e. Test sample added standard solution f. Placebo added standard solution g. Forced degradation of test sample at 80°C h. Forced degradation of test sample under light for 24 hours i. Standard and test solutions (overlay report)

- **System suitability**

Value RSD% of retention time and peak area ≤ 2%, resolution between peaks ≥ 1.5, tailing factor between 0.8 - 1.5 and number of theoretical plates larger than 2000.

Table 3. Results of System Suitability*

Compound	Retention time	% RSD of retention time	%RSD of area	Tailing factor	Theoretical Plates
AML	9.345	0.28	0.32	1.1	43702
OLM	12.889	0.30	0.22	1.0	37122
HCT	5.198	0.27	0.32	1.1	7466

**Average of 6 reading.*

- **Specificity**

All peaks were clearly separated, achieving peak purity (Figure 2), impurity peaks generated under harsh conditions do not overlap with the main peaks.

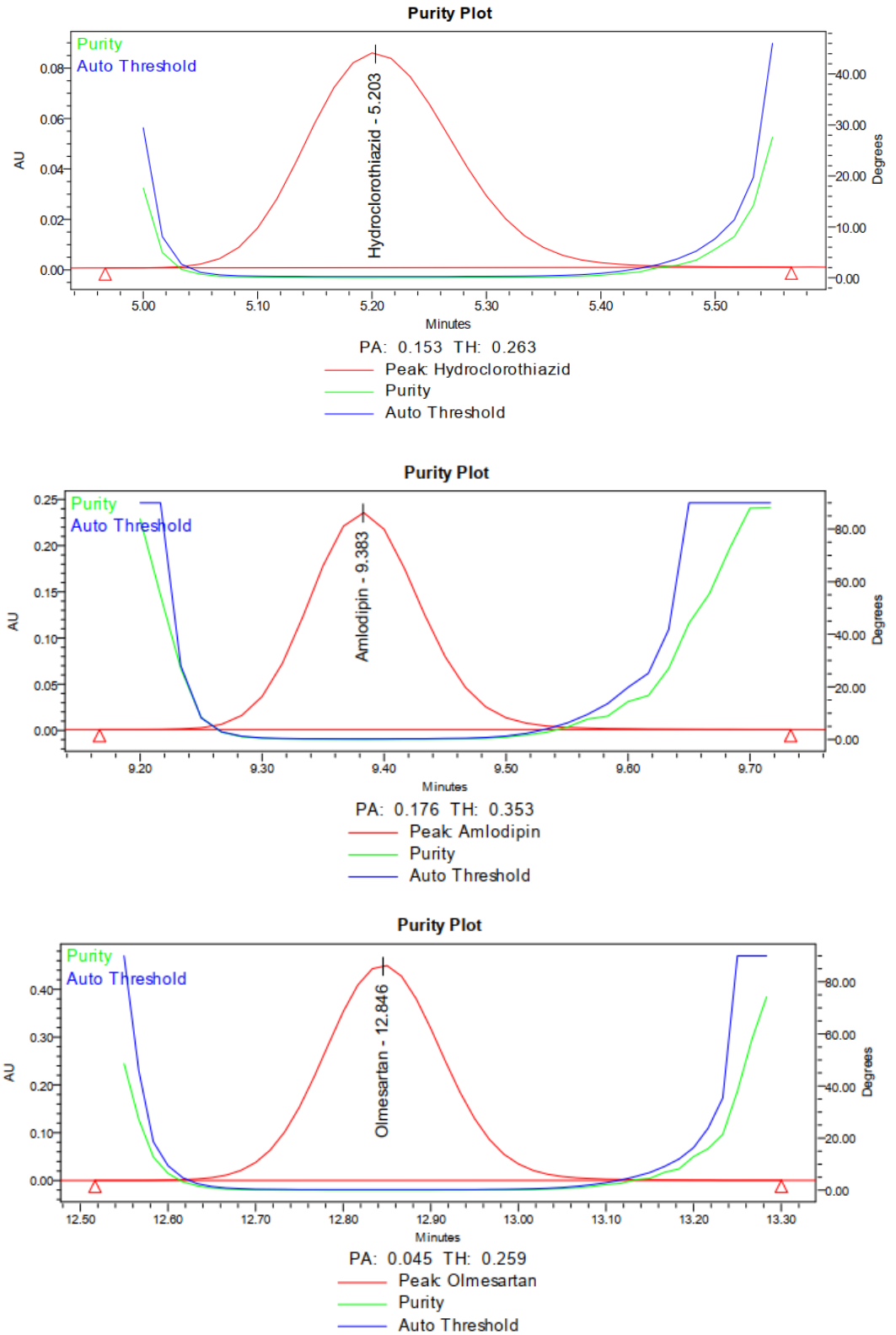


Figure 3. Purity of the peaks in the standard solution mixture

• **Linearity range**

Correlation coefficient R ≥ 0.999, range of HCT is 62.5 - 187.5, AML 34.65 - 103.95, OML 100 - 300 ppm.

• **Accuracy**

Recovery rates of all three substances were in the range of 98-102%.

Table 7. Recovery rates

Excess drug added to the analyte (%)	AML recovery (%)	Mean recovery (%)	Mean RSD %
80	99.88		
100	99.38	99.69	0.27
120	99.80		
Excess drug added to the analyte (%)	**OLM recovery (%)**	**Mean recovery (%)**	**Mean RSD %**
80	99.64		
100	100.14	100.19	0.58
120	100.79		
Excess drug added to the analyte (%)	**HCT recovery (%)**	**Mean recovery (%)**	**Mean RSD %**
80	100.24		
100	100.87	100.52	0.32
120	100.44		

• **Precision**

Precision includes repeatability and intermediate precision with RSD values not more than 2%.

Table 4. Precision Results*

Compound	Repeatability %	%RSD	Intermediate precision	%RSD
AML	100.41	0.92	100.06	0.41
OLM	100.52	0,37	100.35	0.34
HCT	100.13	0,63	99.96	0.62

**Average of 6 reading.*

• **Robustness**

System suitability of the standard solution with satisfactory chromatographic parameters when changing the pH of the buffer solution from 2.5 to 3.5.

4. DISCUSSIONS

In reversed-phase chromatography, the ability to separate analytes depends on many factors such as the nature of analytes, the type of stationary phases, the nature of mobile phases, mobile phase ratios, flow rates, column temperature,…. Therefore, the investigation process starts from testing different mobile phases with different ratios to find the appropriate solvent and ratio to separate the substances.

The project started with the mobile phase system because acetonitrile - phosphoric acid 0.01% because this is an simple, acidic, polar solvent system. Investigation results show that mobile phase composition greatly affects retention time, resolution coefficient, and tailing factor on the chromatogram. Specifically, when reducing the proportion of organic solvent (it means increasing the polarity of the mobile phase), the substances tend to elute more slowly, the retention time increases, the substances change from not separated to separated on the chromatogram and vice versa. Because when increasing or decreasing the proportion of organic solvent, the affinity with the stationary and mobile phases of the substances changes. Especially, when increasing mobile phase polarity, the affinity with the reverse phase is greater, so the substances tend to better distribute in the reverse phase, which leads to slower elution and therefore longer retention time and vice versa.

HCT has pKa value = 7.9; in solution phosphoric acid 0.01% (pH = 3), HCT exists mainly in ionic form, which reduces the interaction of this substance with the stationary phase, resulting in early elution time. Amlodipine has a pKa value = 9.26, so in solution phosphoric acid 0.01% (pH = 3) it also exists mainly in ionic form so it elutes early. OLM has a pKa value = 4.27. In an acidic environment (pH 3), OLM is mostly in non-ionized molecular form, increasing its ability to interact with the stationary phase. [13] In addition, the analytes are not neutral substances, so the retention time is influenced by pH of mobile phase, so when changing the mobile phase ratio, leading to a difference in the order of peaks appearing. Besides, when investigating in this solvent system, the peak shape of amlodipine is abnormal, the peak shape is wide. Therefore, change the phosphoric acid-water into buffer to stabilize the pH, ensuring the stability of the analysis.

When carrying out analysis with the mobile phase system acetonitrile - triethylamine buffer pH 3, the peaks were completely separated, the parameters of tailing factor, number of theoretical plates, resolution coefficient were all met. However, in isocratic mode the HCT peak is not pure or the analysis time is too long. Therefore, the gradient elution program is set up with 3 main stages, the first stage is gradually increasing the proportion of organic solvent, the second stage is the isocratic stage and the third stage is gradually decreasing the organic solvent proportion in original proportions. In gradient program 1, the peaks were completely separated, the retention time was reasonable, the tailing factor was satisfactory, the number of theoretical plates increased significantly, however the HCT peak did not reach purity on the chromatogram. Therefore, gradient program 2 was established with a change in phase 1, which had a longer period of change in the mobile phase ratio, but still no significant change. Continue to investigate the gradient program 3 with a wider range of mobile phase ratio by reducing the organic solvent ratio in the early stage. In this program, all peaks are pure on the chromatogram, parameters of resolution coefficient, tailing factor, number of theoretical plates are all met, and analysis time is reasonable (< 15 minutes). The order of the peaks is HCT (5.127), AML (9.279), OLM (12.889).

This is the first study in Vietnam to simultaneously quantify the three active ingredients AML, OLM, and HCT in tablet preparations using high-performance liquid chromatography. Compared to Janhavi R Rao's study mobile phase containing buffered salt was used [3], in this study we have improved by using evaporation buffer containing triethylamine. The quatitative procedure uses triethylamine buffer instead of phosphate buffer because triethylamine buffer is an organic buffer that helps avoid corrosion of the chromatography system, reduces column and chromatography system washing time, and prolongs the life of the chromatography column more than using inorganic buffer. In addition, using triethylamine buffer avoids risk of salt precipitation in the HPLC system like when using inorganic salt buffer.

5. CONCLUSION

The procedure for simultaneous quantification of HCT, AML and OLM in tablet preparations using high performance liquid chromatography with PDA detector has been developed and validated according to ICH guidelines. The procedure meets the requirements for system suitability, specificity, accuracy, precision, linearity and robustness.

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