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LC and LC–MS methods for the investigation of polypills for the treatment of cardiovascular diseases
Part 1. Separation of active components and classification of their interaction/degradation products

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Abstract

"Polypill" is a fixed-dose combination (FDC) containing three or more drugs in a single pill. The same is under development for the treatment and prevention of cardiovascular diseases. In the present study, gradient LC methods were developed for simultaneous determination of the possible components of a polypill, *i.e.*, lisinopril, aspirin and one each among atenolol/hydrochlorothiazide and atorvastatin/simvastatin/pravastatin, in the presence of a total of 13 major interaction/degradation products. The drugs and the products were well separated using a reversed-phase (C-8) column and a mobile phase comprising of acetonitrile: phosphate buffer (pH 2.3). Other HPLC parameters were flow rate, 1 ml/min; detection wavelength, 210 nm; column oven temperature, 60 °C; and injection volume, 5 μl. The methods were validated for linearity, precision, accuracy, and specificity. These were further modified to make them compatible for LC–MS studies by removal of the phosphate buffer and adjustment of pH by formic acid. The suitability of the methods for LC–MS studies was established by matching the theoretical mass values of the drugs with those obtained experimentally. These methods were used to determine mass values of the major interaction/degradation products, which helped to know the source of their origin.
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Keywords: Atenolol; Hydrochlorothiazide; Lisinopril; Aspirin; Atorvastatin; Simvastatin; Pravastatin; HPLC; LC–MS; Validation

1. Introduction

Cardiovascular diseases (CVDs) are becoming the number one cause of death globally. More people die annually from CVDs than from any other causes [1–3]. Patients at high cardiovascular risk are better benefited from a combination of aspirin, antihypertensive agents, lipid-lowering drugs, and possibly folic acid. As the number of medications that a patient requires increases, adherence and compliance to therapy are likely to decrease [4]. Hence, to avoid these problems, a "polypill" has been proposed as a solution and strategy to fight the disease [5]. "Polypill" is a FDC containing three or more drugs in a single pill for the treatment of CVDs [6]. Subsequent studies have indicated that reduction in the risk of CVDs by lowering serum homocysteine (with folic acid) is largely observational [4,5,7,8], hence its presence in the polypill may be avoided.

Although the term "polypill" has been in use since the publication of the Wald–Law paper [5], drug formulations with multiple active ingredients and the problems associated with them are not new, e.g., multivitamin preparations [9]. However, not much pharmaceutical work is yet reported in the literature on the polypill for CVDs, as it is a new strategy. Even no analytical method existed for the simultaneous determination of possible components of polypill, *i.e.*, atenolol/hydrochlorothiazide, lisinopril, aspirin and atorvastatin/simvastatin/pravastatin, although several HPLC methods have been reported in the literature for their individual analysis [10–21].

Accordingly, the focus of the present study was to develop LC methods for simultaneous determination

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Table 1
Gradient program for atenolol/hydrochlorothiazide, lisinopril, aspirin and simvastatin

Time (min)	A (%)	B (%)	Elution
0.00	5	95	Equilibration
0.01–35.0	5 → 20	95 → 80	Linear gradient
35.0–55.0	20 → 55	80 → 45	Linear gradient
55.0–80.0	55	45	Isocratic
80.0–85.0	55 → 5	45 → 95	Linear gradient
85.0–90.0	5	95	Re-equilibration

Table 2
Gradient program for atenolol/hydrochlorothiazide, lisinopril, aspirin and atorvastatin

Time (min)	A (%)	B (%)	Elution
0.00	5	95	Equilibration
0.01–35.0	5 → 20	95 → 80	Linear gradient
35.0–50.0	20 → 45	80 → 55	Linear gradient
50.0–65.0	45	55	Isocratic
65.0–70.0	45 → 50	55 → 50	Linear gradient
70.0–80.0	50	50	Isocratic
80.0–85.0	55 → 5	45 → 95	Linear gradient
85.0–90.0	5	95	Re-equilibration

of the proposed drugs, *i.e.*, lisinopril, aspirin and one each among atenolol/hydrochlorothiazide and atorvastatin/simvastatin/pravastatin, and to extend the same to stability samples to resolve major interaction/degradation products. Another endeavour was to establish the total number of products formed in specific combinations. It was also planned to modify the developed methods for LC–MS studies, with an objective to determine the mass and to establish the origin of the major interaction/degradation products.

2. Experimental

2.1. Materials

Pure drugs were obtained as gift samples from Dr. Reddy's Laboratories Ltd., Hyderabad, Andhra Pradesh, India. HPLC grade acetonitrile was purchased from J.T. Baker (Mexico City, Mexico). Ultra pure water was obtained from a water purification unit (Elga Ltd., Bucks, England). Buffer materials and all other chemicals were of analytical-reagent grade.

Table 3
Gradient program for atenolol/hydrochlorothiazide, lisinopril, aspirin and pravastatin

Time (min)	A (%)	B (%)	Elution
0.00	5	95	Equilibration
0.01–35.0	5 → 20	95 → 80	Linear gradient
35.0–65.0	20 → 50	80 → 50	Linear gradient
55.0–80.0	50	50	Isocratic
80.0–85.0	50 → 5	50 → 95	Linear gradient
85.0–90.0	5	95	Re-equilibration

Table 4
Linearity data for the drugs ($n = 3$)

Drug	Concentration range (μg/ml)	Equation of regression line	R^2 value
ATE	25–250	$y = 6976x - 359$	0.999
HYD	25–250	$y = 19083x - 18351$	0.999
LIS	25–250	$y = 9584x + 13020$	0.999
ASP	25–250	$y = 4508x - 18386$	0.999
SIM	25–250	$y = 3786x + 62980$	0.999
ATR	25–250	$y = 20914x + 7233$	0.999
PRA	25–250	$y = 56788x + 61794$	0.999

Key—ATE: atenolol; HYD: hydrochlorothiazide; LIS: lisinopril; ASP: aspirin; SIM: simvastatin; ATR: atorvastatin; and PRA: pravastatin.

2.2. Equipment

The HPLC system consisted of an on-line degasser (DGU-14A), low-pressure gradient flow control valve (FCV-10AL$_{VP}$), solvent delivery module (LC-10AT$_{VP}$), auto injector (SIL-10AD$_{VP}$), column oven (CTO-10AS$_{VP}$), photo-diode array (PDA) detector (SPD-M10A$_{VP}$), system controller (SCL-10A$_{VP}$) and CLASS-VP software (all from Shimadzu, Kyoto, Japan). The LC–MS system consisted of an HPLC (1100 series, Agilent Technologies, Waldbronn, Germany) and MicrOTOF-Q mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with ESI source (G-1948A). The LC part comprised of an on-line degasser (G1379A), binary pump (G131A), auto injector (G1313A), column oven (G1316A) and diode array detector (G1315B). The system was controlled by combination of Hyphenation Star (Version 3.1) and MicrOTOF Control (Version 2.0) software. The data were processed by DataAnal-

Table 5
Intra- and inter-day precision studies ($n = 3$)

Drug	Added (μg/ml)	Found ± S.D. (μg/ml), R.S.D. (%)	
		Intra-day precision	Inter-day precision
ATE	50	49.33 ± 0.68, 0.92	48.97 ± 0.07, 0.14
	100	100.94 ± 0.06, 1.39	101.04 ± 0.26, 0.26
	200	201.21 ± 0.35, 0.17	201.93 ± 0.42, 0.21
HYD	50	50.61 ± 0.31, 0.61	50.79 ± 0.15, 0.30
	100	101.34 ± 0.98, 0.97	101.25 ± 0.77, 0.76
	200	200.33 ± 0.89, 0.44	200.43 ± 0.48, 0.24
LIS	50	48.64 ± 0.34, 0.71	48.57 ± 0.25, 0.52
	100	102.29 ± 0.39, 0.38	102.28 ± 0.34, 0.33
	200	199.94 ± 1.23, 0.61	200.01 ± 1.47, 0.73
ASP	50	49.60 ± 0.52, 1.06	49.36 ± 0.30, 0.62
	100	97.35 ± 0.04, 0.04	98.14 ± 1.02, 1.04
	200	200.17 ± 0.70, 0.34	198.29 ± 2.41, 1.21
SIM	50	51.22 ± 0.33, 0.64	51.54 ± 0.30, 0.59
	100	98.65 ± 0.12, 0.12	98.69 ± 0.24, 0.24
	200	201.42 ± 0.59, 0.29	201.92 ± 0.60, 0.29
ATR	50	50.54 ± 0.26, 0.52	50.46 ± 0.40, 0.80
	100	97.38 ± 0.05, 0.05	97.58 ± 0.24, 0.24
	200	199.57 ± 0.74, 0.37	200.62 ± 1.68,0.83
PRA	50	49.57 ± 0.09, 0.18	49.73 ± 0.15, 0.30
	100	100.77 ± 0.22, 0.22	101.01 ± 0.20, 0.20
	200	199.04 ± 0.65, 0.32	198.51 ± 1.54, 0.77

Key: same as in Table 4.

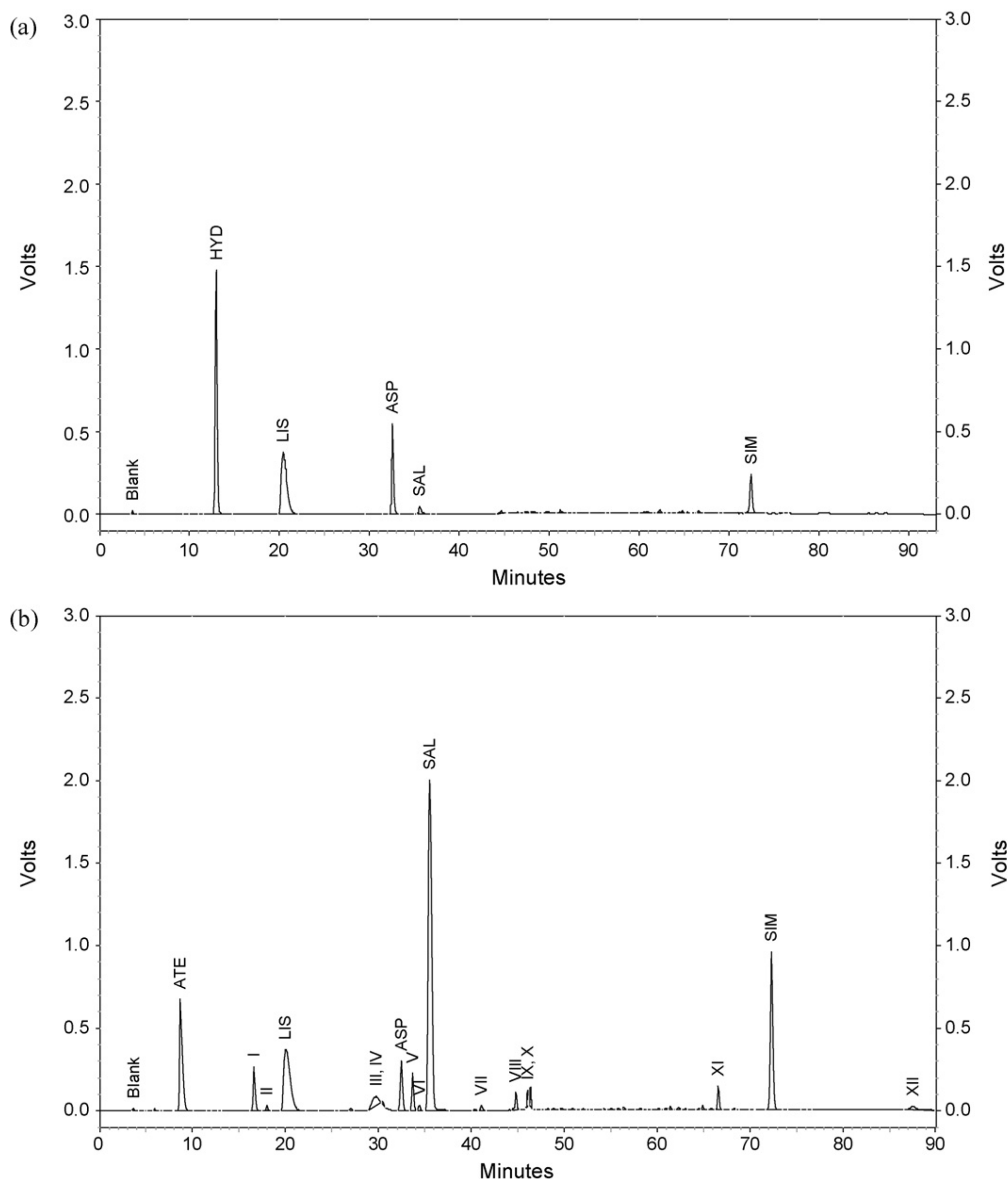

Fig. 1. Chromatograms showing separation of hydrochlorothiazide, lisinopril, aspirin and simvastatin (a) and atenolol, lisinopril, aspirin and simvastatin (b) from their degradation products formed during accelerated stability studies. HYD: hydrocholorothiazide; ATE: atenolol; LIS: lisinopril; ASP: aspirin; SAL: salicylic acid; SIM: simvastatin; I–XII: major degradation products.

ysis (Version 3.3). The separations were achieved on a C-8 (250 mm × 4.6 mm i.d., particle size 5 μm) column (Supelco Discovery, Bellefonate, PA, USA). Stability samples were generated in humidity (KBF720, Binder, Germany) chamber set at 40 ± 1 °C/75% RH ± 3% RH. Other equipments used were sonicator (Branson Ultra-sonic Corporation, Danbury, CT, USA), analytical balance (Mettler Toledo, Schwerzenbach, Switzerland) and auto pipettes (Eppendorf, Hamburg, Germany).

2.3. Degradation studies

All the seven drugs individually, *i.e.*, lisinopril, aspirin, atenolol, hydrochlorothiazide and simvastatin/atorvastatin/pravastatin (50 mg each) and their combinations in the same quantities (total six in numbers) were accurately weighed and transferred to 15 ml glass vials. The same were directly exposed for 90 d in a stability chamber set at 40 ± 1 °C/75% RH ± 3% RH to induce interaction and degradation of the drugs. Samples were withdrawn after 90 d and dissolved in HPLC grade methanol. The resultant solutions were subjected to HPLC studies.

2.4. Development of LC methods

Due to multiple drugs in combination and anticipated multiple interaction/degradation products, the separations were achieved by gradient elution using acetonitrile: phosphate buffer (10 mM, potassium dihydrogen orthophosphate, pH 2.3) as the mobile phase. It was filtered through 0.45 μm nylon filter and degassed before use. The injection volume was 5 μl and flow

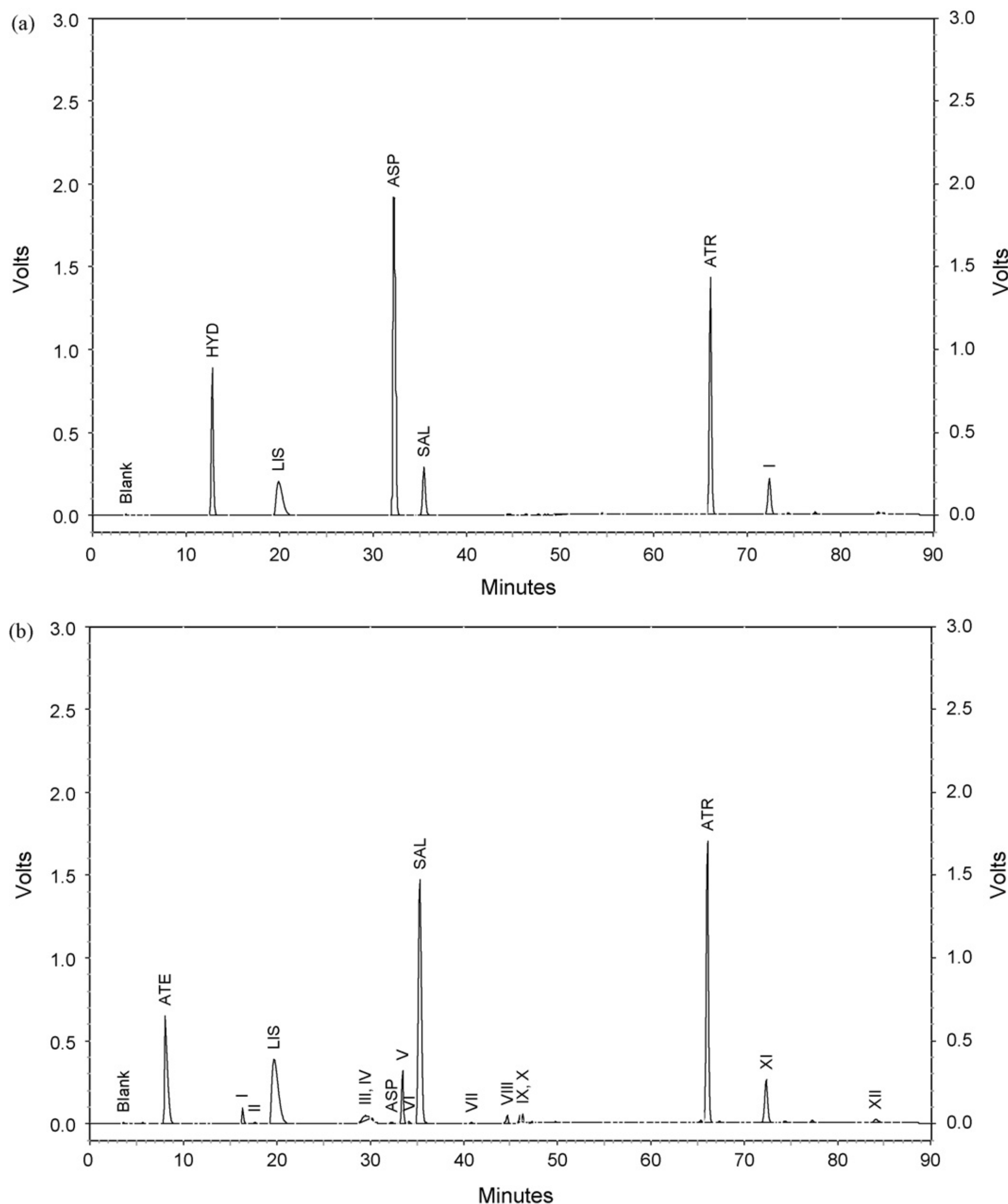

Fig. 2. Chromatograms showing separation of hydrochlorothiazide, lisinopril, aspirin and atorvastatin (a) and atenolol, lisinopril, aspirin and atorvastatin (b) from their degradation products formed during accelerated stability studies. ATR: atorvastatin; remaining same as Fig. 1. *Note*: degradation product I in (a) is same as XI in (b).

rate was 1 ml/min. The detection was carried out at 225 nm and column oven temperature was set at 60 °C.

2.5. Validation of the methods

The above-given methods utilizing a mobile phase containing phosphate buffer was validated for linearity, precision (inter-day, intra-day and intermediate precision), accuracy and specificity. Initially, system suitability was determined with respect to resolution among the active components and USP tailing factor. Standard plots were then constructed for all the drugs in the range of 25–250 μg/ml. The experiment was repeated thrice on the same day and on three consecutive days to determine intra- and inter-day precision, respectively. The intermediate precision of the method was determined by repeating the experiment on two different columns. Accuracy was determined by analyzing the degraded samples of four-drug mixtures generated for method development with three known concentrations of the drugs. Further, specificity of the method was assessed by observing the resolution factor of the drug peaks from nearest resolving peaks. The peak purity of all the drug peaks was determined using a PDA detector.

2.6. LC–MS studies

For LC–MS spectral studies, same methods were used as that of HPLC, except replacement of buffer with water and adjustment of pH using formic acid. The change was necessary because

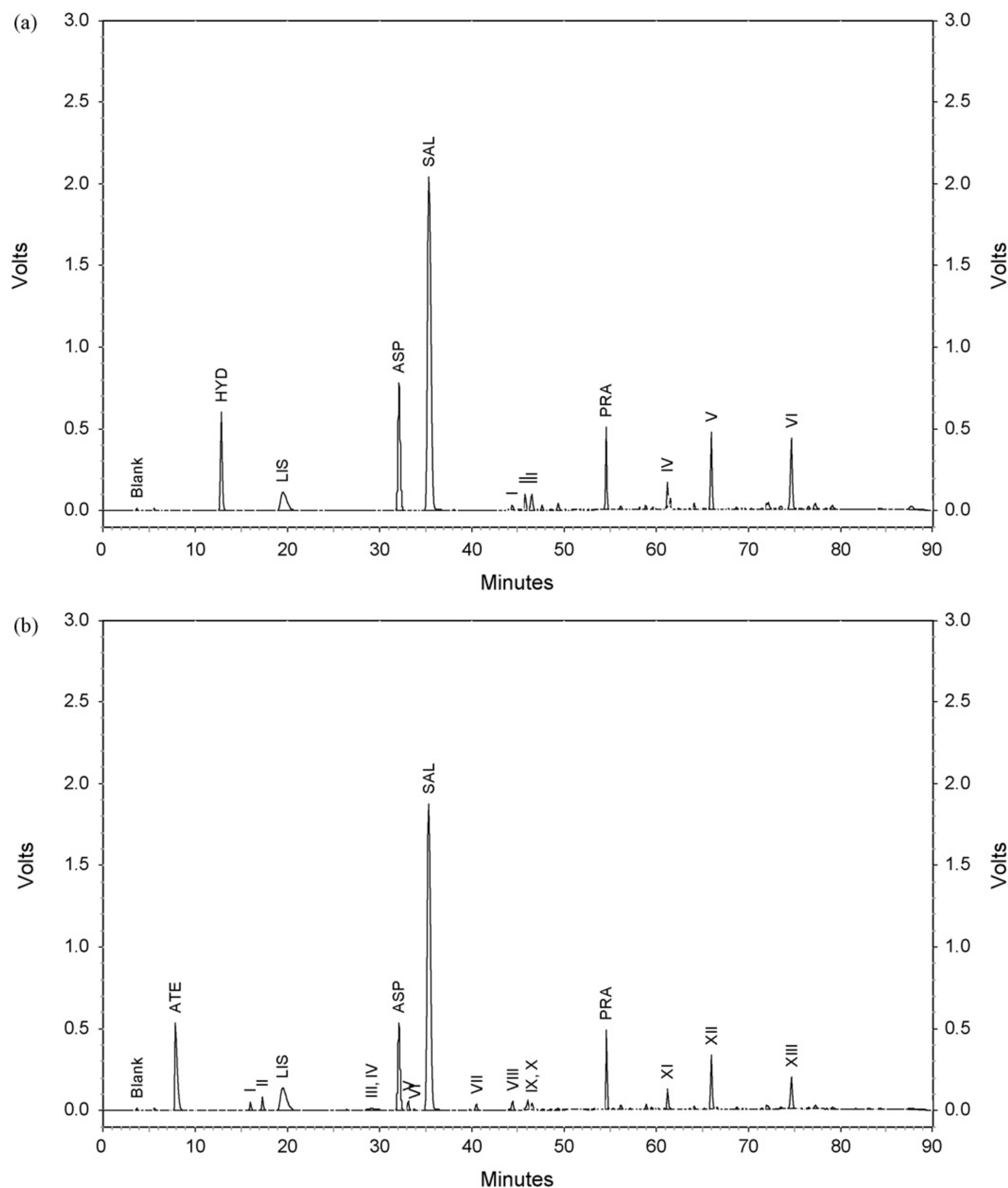

Fig. 3. Chromatograms showing separation of hydrochlorothiazide, lisinopril, aspirin and pravastatin (a) and atenolol, lisinopril, aspirin and pravastatin (b) from their degradation products formed during accelerated stability studies. PRA: pravastatin; remaining same as Fig. 1. *Note*: products I–VI in (a) correspond to VIII–XIII in (b).

of the intolerance of mass ionizer for non-volatile buffer salts. The LC–MS method was not fully validated due to anticipated qualitative use.

The mass spectra were collected in the mass range of 50–3000 amu in either positive or negative ESI ionization modes, depending upon nature of the drugs and interaction/degradation products.

3. Results and discussion

3.1. Development and optimization of the stability-indicating HPLC methods

The gradient HPLC programs for various drug combinations are listed in Tables 1–3. The methods were optimized for the separation of various drug combinations and major interaction/degradation products. Initial program of the gradient for the separation of atenolol/hydrochlorothiazide, lisinopril, and aspirin was the same, whereas different gradients were required in later part for achieving optimum separation of late resolving statins. These were then applied to degraded samples and optimized, as necessary. Figs. 1–3 show the chromatographic separation of all the drugs and their major interaction/degradation products, as obtained by the use of methods listed in Tables 1–3.

3.2. Validation of the developed methods

The system suitability results for both resolution and peak tailing were well within the limits. A linear response was

Table 6
Intermediate precision studies

Drugs	Retention time (R_T)	
	Analyst 1 and instrument 1	Analyst 2 and instrument 2
ATE	~8.0	~7.5
HYD	~13.0	~12.5
LIS	~20.0	~19.0
ASP	~32.0	~31.5
SIM	~72.0	~ 71.5
ATR	~65.0	~ 64.0
PRA	~54.0	~ 54.5

Key: same as in Table 4.

established for all the drugs in the concentration range of 25–250 μg/ml (Table 4). Table 5 lists the relative standard deviation (R.S.D.) data obtained on analysis of the samples on the same day ($n=3$) and on consecutive days ($n=3$). The R.S.D. values were <1% and <2% for intra- and inter-day studies, respectively, demonstrating that the method was sufficiently precise. Even intermediate precision was established for the method, as almost similar resolution behaviour was observed on repeating the experiment on two different HPLC systems and by two different analysts (Table 6). Table 7 shows that recovery of the added drug, obtained from the difference between peak areas of fortified and unfortified degraded samples of all the four-drug mixtures, was satisfactory at all the tested concentrations. As shown in Figs. 1–3, the methods had sufficient specificity as all the drugs were well separated from one another as well as from their interaction/degradation products, with the resolution factor being >2 in all cases. All the drug peaks were pure, which was proved through PDA purity studies. Data of peak purity index and single point threshold values are given in Table 8.

Table 8
PDA peak purity parameters for all the drugs

Drug	Peak purity index	Single point threshold
ATE	1.000	0.998
HYD	1.000	0.998
LIS	0.999	0.998
ASP	0.996	0.995
SIM	1.000	0.998
ATR	1.000	0.998
PRA	1.000	0.998

Key: same as in Table 4.

3.3. Interaction/degradation behaviour and number of products formed

The individual drugs did not degrade significantly in the solid state. However, several interaction/degradation products were generated when other drugs were co-present. As evident from Figs. 1–3, overall degradation was less when hydrochlorothiazide was present in the combination, while it was higher in the combinations containing atenolol. In case of combinations containing hydrochlorothiazide, lisinopril, aspirin and simvastatin (Fig. 1a), no major product was formed, whereas total 12 products were formed when atenolol was present in the combination (Fig. 1b). Similarly, in case of hydrochlorothiazide, lisinopril, aspirin and atorvastatin (Fig. 2a) only one major degradation product was formed, whereas 12 products were formed in the presence of atenolol in the combination (Fig. 2b).

Table 7
Recovery studies

Drug	Added concentration (μg/ml)	Measured concentration (μg/ml)	% recovery	Mean % recovery
	75	74.92	99.89	
ATE	200	201.70	100.75	100.50 ± 0.52%
	225	226.70	100.85	
	75	74.30	99.07	
HYD	200	199.80	99.90	99.71 ± 0.56%
	225	225.38	100.16	
	75	75.01	100.02	
LIS	200	199.52	101.76	100.87 ± 1.08%
	225	228.96	99.76	
ASP	75	75.89	101.18	
	200	197.16	98.58	99.86 ± 1.30%
	225	224.60	99.82	
	75	75.18	100.24	
SIM	200	199.47	99.73	99.97 ± 0.25%
	225	224.83	99.92	
	75	75.25	100.34	
ATR	200	200.80	100.40	100.16 ± 0.45%
	225	224.94	99.75	
	75	75.55	100.73	
PRA	200	199.35	99.67	100.20 ± 0.52%
	225	225.43	100.19	

Key: same as in Table 4.

Table 9
Observed *m*/*z* values for the $[M+H]^+$ or $[M-H]^-$ ions along with major fragments of the drugs

Drug	Observed *m*/*z* value	ESI	Major fragments			
			I	II	III	IV
ATE	267.20	+ve	225.15	208.12	190.11	145.08
HYD	296.00	−ve	268.98	–	–	–
LIS	406.27	+ve	309.21	291.20	263.20	246.17
ASP	179.05	−ve	157.00	141.03	137.04	–
SIM	419.32	+ve	285.22	243.20	225.19	199.17
ATR	559.28	+ve	466.22	440.24	–	–
PRA	447.25[a]	+ve	305.19	269.17	209.14	199.14
PRA	423.26	−ve	341.12	321.18	303.17	–

Key: same as in Table 4.
[a] $[M+Na]^+$.

In case of pravastatin containing combinations, six products were formed when hydrochlorothiazide was present in the combination (Fig. 3a), whereas a total of 13 products were formed with atenolol (Fig. 3b). This indicated stronger complexity in the presence of pravastatin than other statins, and in the presence of atenolol than hydrochlorothiazide. The catalysis of degradation by atenolol may be due to its imparting an alkaline microenvironment, which is under investigation.

3.4. LC–MS studies

Almost similar resolution behaviour was obtained by simple replacement of phosphate buffer with water, and adjustment of pH by formic acid. In comparison to retention times obtained with LC-PDA in Figs. 1–3, the same for atenolol, hydrochlorothiazide, lisinopril, aspirin, simvastatin, atorvastatin and pravastatin in volatile buffer LC–MS method were around 7, 12, 19, 31, 71, 63, and 54 min, respectively. The retention times for the interaction/degradation products I–X were around 16, 17, 29, 30, 32, 34, 40, 44, 45 and 46 min, respectively. The retention times for products XI and XII in case of combination containing simvastatin were ∼66 and ∼86 min, while the same for products XI and XII for the combination containing atorvastatin were ∼72 and ∼83 min, respectively. The retention times for products XI–XIII for combination containing pravastatin were ∼61, ∼66 and ∼75 min, respectively.

Using the LC–MS method, mass spectra were recorded for the drugs as well as the major interaction/degradation products. Reasonable mass spectra were obtained for atenolol, lisinopril, simvastatin and atorvastatin in positive ESI mode, while hydrochlorothiazide and aspirin ionized better in negative ESI mode. Pravastatin gave satisfactory molecular ion peak in both positive and negative ESI modes. The data for the drugs are included in Table 9. Table 10 shows mass to charge values for interaction/degradation products of the mixtures of atenolol/hydrochlorothiazide, lisinopril and simvastatin (A), atenolol/hydrochlorothiazide, lisinopril and atorvastatin (B) and atenolol/hydrochlorothiazide, lisinopril and pravastatin (C). All the interaction/degradation products gave good mass spectra in positive ESI mode, except for the two products, XII and XIII, related to pravastatin, which showed good mass peaks in negative ESI mode. The mass fragmentation behaviour of interaction/degradation products was largely similar to the drugs, as given in Table 9, on which basis it was concluded that the products I, II, V, VII, IX were related to atenolol, while III, IV, VI, VIII, X were related to lisinopril. In a similar manner, products XI and XII in combination A were related to simvastatin, the same products for B were related to atorvastatin, and prod-

Table 10
Mass to charge (*m*/*z*) values[a] of interaction/degradation products shown in Figs. 1–3

Peak	A	B	C	Comparability among combinations A–C	Product type
I	309.21	309.21	309.21	Similar	Interaction product
II	268.18	268.18	268.18	Similar	–
III	448.28	448.28	448.28	Similar	Interaction product
IV	448.29	448.29	448.29	Similar	Interaction product
V	309.18	309.18	309.18	Similar	Interaction product
VI	388.26	388.26	388.26	Similar	Degradation product
VII	429.24	429.24	429.24	Similar	Interaction product
VIII	430.27	430.27	430.27	Similar	Interaction product
IX	351.21	351.21	351.21	Similar	Interaction product
X	490.30	490.30	490.30	Similar	Interaction product
XI	437.33	541.27	411.23	Different	Degradation products
XII	401.31	523.25	543.28	Different	Degradation products in case of A and B, and interaction product in case of C
XIII	–	–	525.28	–	Interaction product

A–C: *m*/*z* values of interaction/degradation products shown in Figs. 1–3, respectively.
[a] $[M+H]^+$ values for various products, except XII and XIII of combination C where $[M-H]^-$ data are listed.

ucts XI–XIII for C were related to pravastatin. The products with molecular weights lesser than respective drugs or higher by 18 amu (due to addition of water) were categorized as degradation products, whereas those with molecular weight greater than that of the drug by +18 amu were considered as interaction products (Table 10).

4. Conclusions

This study presents simple and validated LC and LC–MS methods for simultaneous estimation of atenolol/hydrochlorothiazide, lisinopril, aspirin and simvastatin /atorvastatin/pravastatin in the presence of their major interaction/degradation products. The given methods can be exploited for drug–drug interaction and stability studies on the polypills.

It is observed that multiple interaction/degradation products (up to 13) are produced in different drug combinations possible for the polypill. These preliminary studies are being extended to assign specific structures to each of the interaction/degradation products by LC–MS-TOF, LC–MS^n and LC–NMR (wherever possible). Detailed results of identification studies will be reported in a future communication.

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