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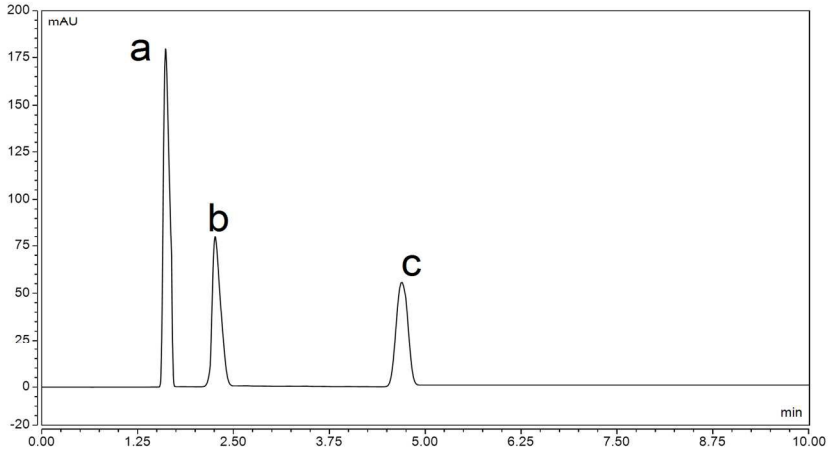

First method for determination of empagliflozin either alone or in combination
First simultaneous separation of empagliflozin, linagliptin and metformin

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UPLC Simultaneous Determination of Empagliflozin, Linagliptin and Metformin New Combinations

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Abstract:

The first UPLC method was developed for simultaneous determination of empagliflozin, linagliptin and metformin hydrochloride in their different combinations as pharmaceutical dosage forms. Chromatographic separation was achieved on a Symmetry® Acclaim™ RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 µm) applying an isocratic elution based on potassium dihydrogen phosphate buffer pH (4) - methanol (50:50, *v/v*) as a mobile phase. Linearity, accuracy and precision were found to be acceptable over the concentration ranges of 1-32 $\mu g ml^{-1}$, 0.5-16 $\mu g ml^{-1}$ and 1-100 $\mu g ml^{-1}$ for empagliflozin, linagliptin and metformin hydrochloride, respectively. All the variables were studied to optimize the chromatographic conditions. The optimized method was validated and proved to be suitable for the quality control of the mentioned drugs in their different pharmaceutical dosage forms.

Keywords: Empagliflozin; Linagliptin; Metformin hydrochloride; UPLC; pharmaceutical dosage forms.

1. Introduction:

Empagliflozin (EGN), (2S,3R,4R,5S,6R) -2- [4-chloro-3-[[4-[(3S)-oxolan-3-yl] oxyphenyl] methyl] phenyl] -6- (hydroxymethyl) oxane-3,4,5-triol (Fig. 1-a) is an inhibitor of the sodium glucose co-transporter-2 (SGLT-2), which accounts for about 90 percent of glucose reabsorption into the blood [1]. Linagliptin (LGP), 8- [(3*R*) -3-aminopiperidin-1-yl] -7-(but-2-yn-1-yl)-3-methyl-1- [(4-methylquinazolin-2-yl) methyl] -3,7-dihydro-1*H*-purine-2,6-dione] (Figure 1-b) belongs to dipeptidyl-peptidase-4 inhibitor class which stimulates glucose-dependent insulin release [2]. Metformin hydrochloride (MET), *N*,*N*-dimethylimidodicarbonimidic diamide (Fig.1-c) is a biguanide hypoglycemic drug exerts its effect mainly by increasing peripheral utilization of glucose [3].

To the best of the author knowledge, no published methods are available for determination of EGN either alone or in combinations in pharmaceutical dosage forms. Literature review showed that few methods were described for determination of LGP in pharmaceutical preparation including spectrophotometry and chromatographic methods [4-9]. In addition, numerous analytical methods were reported for determination of MET

in pharmaceutical preparation including spectrophotometry and chromatographic methods [10-25].

The aim of the new proposed UPLC method is to present the first method for simultaneous determination of EGN, LGP and MET in bulk and in their different combinations as pharmaceutical dosage forms. Furthermore, it is the first method for determination of empagliflozin either alone or in combinations. In addition, the established UPLC method has many advantages over the routine HPLC methods reported for LGP and MET as UPLC is more economic consuming less organic solvent and less time. Finally, the new developed UPLC method has major advantages in comparison to the reported methods for LGP and MET [4-25] including simultaneous determination of EMP with LGP and MET, less retention times, enhanced resolution, better sensitivity and simple mobile phase. Most of the advantages may be attributed to moving from HPLC to UPLC.

Figure 1

2. Experimental

2.1. Instrumentation

The liquid chromatography consisted of a Thermo Fisher UPLC SYSTEM Model Ultimate 3000 Complete Ultra High Performance Liquid Chromatography (USA). For the UPLC system, a Symmetry® Acclaim™ RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 µm) (USA) was used. The system was equipped with a Diode Array detector (DAD-3000RS, USA) and an autosampler (WPS-3000TRS, Thermo scientific, USA). An Elmasonic S 60 H (Germany) was used for the degassing of the mobile phases. Jenway

digital pH meter was used to adjust and determine the hydrogen ion concentration (pH) of the buffer solutions.

2.2. Reagents and reference samples

Pharmaceutical grade EGN and LGP certified to contain 99.70 % and 99.90 %, respectively, Jardiance® tablets nominally containing 25 mg of EGN per tablet, Tradjenta® tablets nominally containing 5 mg of LGP per tablet, Jentadueto® tablets nominally containing 2.5 mg of LGP and 500 mg of metformin hydrochloride per tablet, Synjardy® tablets nominally containing 12.5 mg of EGN and 850 mg of MET per tablet and Glyxambi® tablets nominally containing 5 mg of linagliptin and 10 mg of empagliflozin per tablet were supplied from Boehringer Ingelheim pharmaceutical company (Germany). Metformin hydrochloride, certified to contain 99.80 % was supplied from Chemical Industries Development (CID) Co. (Giza, Egypt). Glucophage® tablets nominally containing 500 mg metformin per tablet were supplied from Merck Serono (Egypt).

HPLC grade methanol was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Orthophosphric acid (85%) was purchased from VWR Chemicals (Pool, England). HPLC grade potassium dihydrogen phosphate was purchased from Sigma Aldrich (Deisenhofen, Germany). Bi-distilled water was produced in-house (POLNA, DE 10, Poland). PTFE Membrane Filter, 47 mm, 0.20 µm (100/pk), (UK) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise.

2.3. Standard stock solutions

Standard stock solutions of EGN, LGP & MET (1 mg mL^{-1}) was prepared by dissolving 100 mg of the drug in methanol using a 100 mL volumetric flask and completing to volume with methanol, sonicated for ten minutes and then the required concentrations were prepared by serial dilutions in the mobile phase.

2.4. Working solutions

Working solution of EGN (40 µg mL^{-1}) was prepared by serial dilution of 4 ml of its stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase. Working solution of LGP (20 µg mL^{-1}) was prepared by serial dilution of 2 ml of its stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase. While the working solution of MET (100 µg mL^{-1}) was prepared by serial dilution of 10 ml of its stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase.

2.5. Chromatographic conditions

Chromatographic separation was achieved on a Symmetry® Acclaim™ RSLC 120 C_{18} column (100 mm × 2.1 mm, 2.2 µm) applying an isocratic elution based on potassium dihydrogen phosphate buffer pH (4) - methanol (50:50, *v/v*) as a mobile phase. The ultraviolet detector was operated at 225 nm. The buffer solution was filtered through 0.2 µm membrane filter and degassed for 30 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 0.4 mL min^{-1}. The column temperature was adjusted to 50° C and the injection volume was 10 µL.

2.6. Sample preparation

Twenty tablets of Jardiance® , Tradjenta®, Glucophage®, Jentadueto®, Synjardy® and Glyxambi® were separately weighed, powdered and mixed in a mortar. An accurately

weighed amount of the finely powdered Jardiance®, Tradjenta® and Glucophage® tablets equivalent to 10 mg of EGN, LGP and MET, respectively were separately made up to 100 mL with methanol and sonicated to dissolve. Also an accurately weighed amount of the finely powdered Jentadueto® tablets equivalent to 0.5 mg of LGP and 100 mg of MET, Synjardy® tablets equivalent to 1 mg of EGN and 68 mg of MET and Glyxambi® tablets equivalent to 2.5 mg of EGN and 5 mg of LGP were separately made up to 100 mL with methanol and sonicated to dissolve. The solutions were filtered followed by serial dilutions to the required concentrations using the mobile phase for each experiment including the standard addition technique.

2.7. Procedure

2.7.1. Linearity

Accurately measured aliquots of stock solutions equivalent to 10-320 µg, 5-160 µg and 10-1000 µg for EGN, LGP and MET, respectively were transferred separately into a series of 10 mL volumetric flasks, completed to volume with the mobile phase, transferred to the vials of the auto sampler and only ten micro liters were injected. A calibration curve was obtained by plotting Area under the peak (AUP) against concentration (C).

2.7.2. Assay of EGN, LGP and MET in bulk (accuracy),lab prepared mixtures, Jardiance®, Tradjenta®, Glucophage®, Jentadueto®, Synjardy® and Glyxambi® tablets

The procedure mentioned under 2.7.1. was repeated using concentrations equivalent to 5, 10, 15, 20 and 25 µg mL^{-1} EGN and equivalent to 3, 6, 9, 12 and 15 µg mL^{-1} LGP and equivalent to 10, 30, 50, 70 and 90 µg mL^{-1} MET. Also Different ratios of EGN, LGP and MET were prepared in the laboratory prepared mixtures. For the determination of EGN, LGP and MET in Jardiance®, Tradjenta®, Glucophage®,

Jentadueto®, Synjardy® and Glyxambi® tablets, the sample solutions prepared under 2.6. were serially diluted and then the procedure mentioned under 2.7.1. was repeated. The concentrations of the mentioned drugs were calculated using their specified calibration equations.

2.7.3. Precision of the proposed method

Three different concentrations of EGN (16, 20 and 24 μg.mL^{-1}), LGP (8, 10 and 12 μg.mL^{-1}) and MET (40, 50 and 60 μg.mL^{-1}), were analyzed three times, within the same day, using the procedure mentioned under (2.7.1). Also the mentioned concentrations were analyzed on three successive days using the same procedure.

2.7.4. Robustness for the chromatographic method

The flow rate of the mobile phase was changed from 0.4 mL min^{-1} to 0.38 mL min^{-1} and 0.42 mLmin^{-1}. The organic strength was changed by % ± 1. Finally, the value of pH of the phosphate buffer was varied from 4.0 to 3.9 and 4.1.

3. Results and discussion

3.1. Advantages of the new proposed UPLC method

It is the first method for simultaneous determination of EGN, LGP and MET with possible application on six approved dosage forms including EGN (Jardiance®), LGP (Tradjenta®), MET (Glucophage®), LGP & MET (Jentadueto®), EGN & MET (Synjardy®), EGN & LGP (Glyxambi®). Also, to the best of the author knowledge, no published methods are available for sensitive determination of EGN either alone or in combinations in pharmaceutical dosage forms.

In addition, it is the first method that applies UPLC for LGP and MET rather than HPLC with many advantages as UPLC operates at much higher pressures. This ultra pressure ensures the advantages of improved resolution and fewer consumables. One of

the key advantages is resolution as defined by the peak shape. HPLC typically produces broad peaks that skilled operators can characterize very well, including peak heights and peak widths. Another important advantage is faster runtime. The significant reduction in solvent use is another important advantage for UPLC. Not only does the higher pressure system require less solvent, but shorter run times also require less solvent. Moving from HPLC to UPLC means higher resolution coupled with increased throughput analysis, decreased solvent use and decreased cost.

3.2. Method development for the chromatographic method

During the optimization cycle, several columns were tried for the experiment, but the C_{18} showed the best results with good peak intensity, sharp peaks with enhanced resolution and high throughput elution. Using cyano column reversed the elution with less resolution and less symmetric peaks. Various mobile phase compositions, containing different ratios of organic and aqueous phases were tried in an isocratic mode. Methanol was found the optimum for elution process. Besides, different buffers at different pH values were attempted along with methanol. Also the pH value of the buffer was adjusted to be 4.00 to ensure its value below or above the Pk_a values of the studied drugs by more than two as Pk_a of EGN is 12.6 and Pk_a of MET is 12.4 while LGP has two Pk_a values which are 1.9 and 8.6.

UV detection at 225 nm was selected according to UV spectra of the studied drugs and the phosphate buffer showed the optimum conditioning at this lambda rather than acetate, formate and other buffers. Adjusting the flow rate to 0.4 ml/min was crucial for the proposed method to enhance the resolution between the three peaks. Applying flow rate more than 0.4 increased the back pressure of the UPLC system more than 400 psi which is not favorable. The marked increase in the column temperature to 50° C allowed more symmetry of the eluted peaks with less noise and less tailing while increase the

column temperature over 50° C was not applicable to avoid thermal degradation of the processed samples.

3.3. System suitability tests for the UPLC method

System suitability tests were used to verify that; the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The resolution factor shows the accuracy of the quantitative analysis, it is specified to ensure that; closely eluting compounds are resolved from each other, Column efficiency is specified as system suitability requirement as a measure of peak sharpness, which is important for detection of trace compounds, while the tailing factor is a measure of peak symmetry. Different parameters affecting the chromatographic separation were studied, including column efficiency (number of theoretical plates), tailing of chromatographic peak, peak resolution factor and % RSD of the peak area and retention time of six injections as shown in (Table 1).

Table 1

3.4. Method validation

The method was validated according to ICH guidelines [26].

3.4.1. Linearity

In this study; a linear relationship between area under the peak (AUP) and component concentration (C) was obtained for six chosen concentrations of each drug and the regression equations were then computed for the chromatographic method. The linearity of the calibration curves were validated by the high value of correlation coefficient, acceptable values of regression coefficient, standard deviation of the slope and standard deviation of the intercept as shown in (Table 2).

Table 2

3.4.2. Accuracy

Accuracy of the results was calculated by % recovery of 5 different concentrations of each drug. The results including the mean of the recovery and standard deviation are shown in (Table 2).

3.4.3. Precision

3.4.3.1. Repeatability:

Three concentrations of EGN (16, 20 and 24 $\mu g.mL^{-1}$), three concentrations of LGP (8, 10 and 12 $\mu g.mL^{-1}$) and three concentrations of MET (40, 50 and 60 $\mu g.mL^{-1}$), were analyzed three times, within the same day, using the procedure mentioned under (2.7). The % RSD was calculated and found to be less than 2% in the three concentrations, as shown in (Table 2).

3.4.3.2. Intermediate precision:

The above mentioned concentrations were analyzed on three successive days using, the procedure mentioned under (2.7). The % RSD was calculated and the results are shown in (Table 2).

3.4.4. Robustness for the chromatographic method

Robustness was performed by deliberately changing the chromatographic conditions. The most important parameter to be studied was the resolution factor between the two peaks of MET and LGP and also between the two peaks of LGP and EGN. The flow rate of the mobile phase was changed from 0.4 mL min^{-1} to be 0.38 mL min^{-1} and 0.42 mL min^{-1}, where resolution factors obtained were (2.08, 4.81), (1.98, 4.67) and (2.19, 4.94), respectively. Besides, the ratio of methanol was changed from 50% to 49% and 51%, where resolution factors obtained were (2.08, 4.81), (2.13, 4.69) and (2.16,

4.86), respectively. Finally, the value of pH of the phosphate buffer was varied from 4.0 to 3.9 and 4.1, where resolution factors obtained were (2.08, 4.81), (1.90, 4.85) and (2.10, 4.97), respectively. There is no significant difference in the results obtained for all these variations, indicating good robustness of the proposed LC method.

3.4.5. Specificity

Specificity is the ability of the analytical method to measure the analyte response in the presence of interferences including the other drugs or excipients. In the present work, specificity was checked by analyzing 5 different concentrations of each drug in the presence of the excipients of different pharmaceutical dosage forms. The results including the mean of the recovery and standard deviation are shown in (Table 2).

3.4.6. Limit of detection and limit of quantification

Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3.3 and limit of quantification (LOQ) at which S/N is 10 were determined and results are given in (Table 2). Low values of LOD and LOQ indicate sensitivity of the applied method for determination of the mentioned drugs in tablets.

3.4.7. Pharmaceutical dosage forms & standard addition technique

The proposed chromatographic method was successfully applied to different pharmaceutical dosage forms and to check the validity of the proposed method, the standard addition technique was applied by adding different known concentrations of the pure drug (2, 4 and 8 µg mL^{-1} of EGN, 1, 2 and 4 µg mL^{-1} of LGP and 10, 20 and 40 µg mL^{-1} of MET) to different known concentrations of each drug product and the procedure mentioned above were adopted (Figures 2 and 3). The concentrations were calculated using the corresponding regression equations as in table 2.

Figures 2 and 3

3.4.8. Statistical analysis

Statistical analysis of the results obtained by the proposed method and the reference well established HPLC method for determination of LGP and MET [5] was carried out by "SPSS statistical package version 11". The significant difference between the reference method and the described method was tested by (*t*-test) and (F-test) at *P*=0.05 as shown in (Table 3). The tests ascertained that; there was no significant difference among the methods. While no statistical comparison was carried out for EGN as the proposed method is the first method for its determination either alone or in combinations.

Table 3

4. Conclusion

The new proposed method proved to be simple, accurate, precise and reproducible for simultaneous determination of EGN, LGP and MET in bulk and in their different combinations in pharmaceutical dosage forms in a reasonable run time with high throughput analysis. The method was validated showing satisfactory data for all the parameters tested. The developed UPLC method can be conveniently used by quality control laboratories with the advantages of using simple mobile phase, saving time and decreased cost by using less solvent in UPLC.

5. Acknowledgement:

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Table (1): System suitability tests for the proposed UPLC method:

Item	MET		LGP		EGN
N	1584		2614		2874
T	1.02		1.01		1.00
R		2.08		4.81	
% RSD for 6 injections (Peak area)	0.32		0.21		0.19
% RSD for 6 injections (Retention time)	0.18		0.15		0.33

N: Number of theoretical plates, T: Tailing factor,
R: Resolution between two consecutive peaks, % RSD: Percent relative standard deviation

Table (2): Results obtained by the proposed UPLC method for simultaneous determination of EGN, LGP and MET:

Item	MET	LGP	EGN
UPLC-UV detection	225 nm	225 nm	225 nm
Retention time	1.6	2.3	4.7
Linearity	1-100 $\mu g.ml^{-1}$	0.5-16 $\mu g.ml^{-1}$	1-32 $\mu g.ml^{-1}$
Regression equation	AUP = 1.3213 $C_{\mu g/ml}$+ 0.128	AUP = 3.4148 $C_{\mu g/ml}$+ 0.0301	AUP = 4.7462 $C_{\mu g/ml}$+ 0.0481
Regression coefficient (r^2)	1	0.9998	0.9999
Standard deviation of slope	0.012	0.026	0.035
Standard deviation of intercept	0.03	0.08	0.05
Accuracy (mean ± SD)	100.65 ± 1.18	98.88 ± 1.06	99.81 ± 0.90
Dosage forms (mean ± SD)	99.28 ± 0.77	99.62 ± 1.16	99.25 ± 1.23
Drug added (Standard addition)	99.38 ± 0.86	98.64 ± 0.83	100.59 ± 1.33
LOD	0.21 $\mu g.ml^{-1}$	0.12 $\mu g.ml^{-1}$	0.26 $\mu g.ml^{-1}$
LOQ	0.63 $\mu g.ml^{-1}$	0.36 $\mu g.ml^{-1}$	0.78 $\mu g.ml^{-1}$
Intraday % RSD	0.16-0.27	0.22-0.34	0.19-0.28
Interday % RSD	0.12-0.30	0.10-0.26	0.14-0.31

Table (3): Statistical comparison between the results of the proposed UPLC method and the reference method of LGP and MET

Statistical Term	Reference Method LGP [5]	UPLC Method LGP	Reference Method MET [5]	UPLC Method MET
Mean	99.45	98.88	100.40	100.65
S.D.±	1.34	1.06	1.05	1.18
% RSD	1.35	1.07	1.05	1.18
n	5	5	5	5
V	1.8	1.12	1.1	1.39
t (*2.306)		0.75		0.35
F (*6.39)		1.61		1.26

* Figures in parentheses are the theoretical t and F values at (p=0.05).

List of Figure Legends:

Figure (1): Chemical structures of Empagliflozin (a), Linagliptin (b) and Metformin (c).

Figure (2):

a- UPLC chromatogram of a lab prepared mixture of (a) Metformin hydrochloride (20 µg mL^{-1}), (b) Linagliptin (1.5 µg mL^{-1}) and (c) Empagliflozin (1.5 µg mL^{-1}).

b- UPLC chromatogram of Jentadueto® tablet extract in methanol containing (a) Metformin hydrochloride (100 µg mL^{-1}) and (b) Linagliptin (0.5 µg mL^{-1}).

c- UPLC chromatogram of Synjardy® tablet extract in methanol containing (a) Metformin hydrochloride (68 µg mL^{-1}) and (b) Empagliflozin (1 µg mL^{-1}).

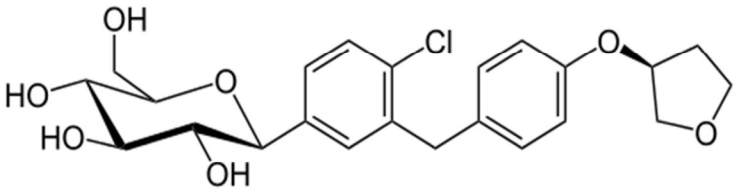
d- UPLC chromatogram of Glyxambi® tablet extract in methanol containing (a) Linagliptin (2.5 µg mL^{-1}) and (b) Empagliflozin (5 µg mL^{-1}).

Figure (3):

a- UPLC chromatogram of Jardiance® tablet extract in methanol containing Empagliflozin (1 µg mL^{-1}) after serial dilution.

b- UPLC chromatogram of Tradjenta® tablet extract in methanol containing Linagliptin (5 µg mL^{-1}) after serial dilution.

c- UPLC chromatogram of Glucophage® tablet extract in methanol containing Metformin hydrochloride (10 µg mL^{-1}) after serial dilution.



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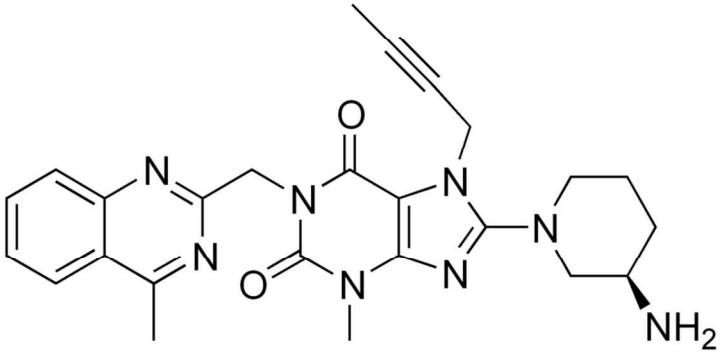

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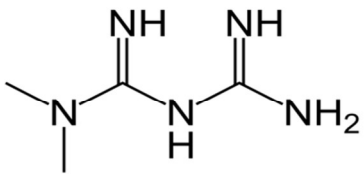

(c)

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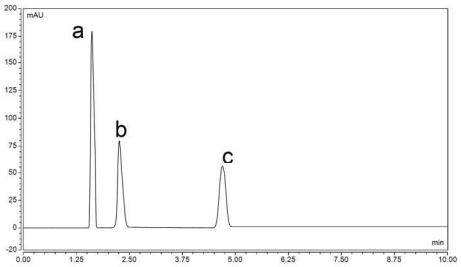

Figure 2 (a)

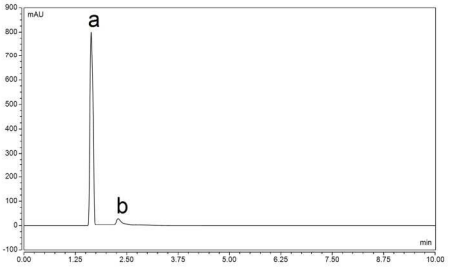

Figure 2 (b)

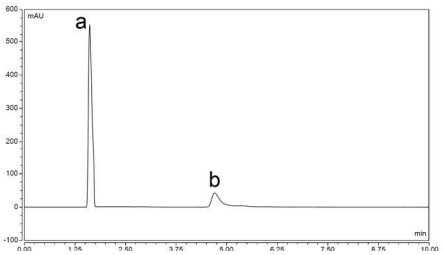

Figure 2 (c)

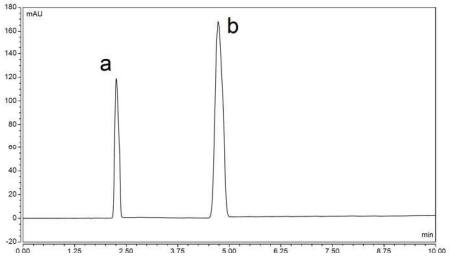

Figure 2 (d)

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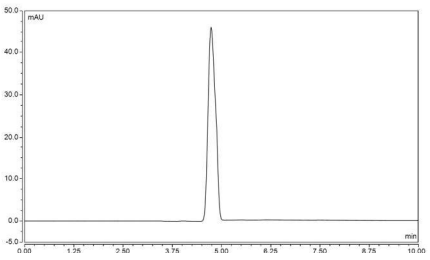

Figure 3 (a)

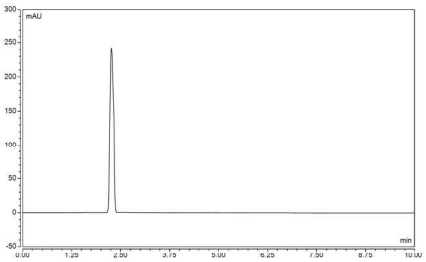

Figure 3 (b)

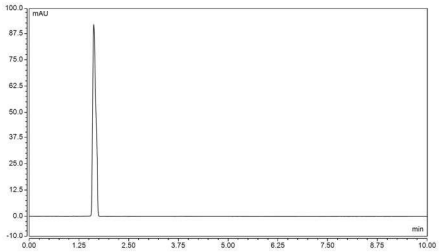

Figure 3 (c)

254x190mm (300 x 300 DPI)