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SIMULTANEOUS DETERMINATION OF PHENYTOIN AND LAMOTRIGINE IN HUMAN PLASMA USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY-TRIPLE QUADRUPOLE MASS SPECTROMETRY.

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A fast, sensitive and selective method for the detection and quantification of Phenytoin (PHT) and Lamotrigine (LTG) in plasma is described using high-performance liquid chromatographic separation with tandem mass spectrometry. Samples were purified using liquid–liquid extraction and separated on a Phenomenex Luna HILIC 3µ (2× 150 mm) column with a mobile phase consisting acetonitril/ ammonium formate (5mM, pH 3.5) (97.5:2.5, v/v). Detection was performed by a triple quadrupole model G6410A mass spectrometer in the MRM mode for PHT and SIM mode for LTG , monitoring the transition of the deprotonated molecular ion for PHT at *m*/*z* 251 to the predominant ions of *m*/*z* 102 and protonated molecular ion for LTG at *m*/*z* 256. The mean recovery was 90% for PHT and 80% for LTG. The LODs for PHT and LTG in human plasma were 5.17 ng/ml and 0.13 ng/ml, respectively. The LOQs for both analytes in human plasma were 17.24 ng/ml and 0.46 ng/ml, respectively. In addition, this study shows that the internal standard at MRM mode could not be used for calculation of peak ratio of compound that has determined at SIM mode and vice versa.

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Keywords: Hilic, LC MS MS, Phenytoin, Lamotrigine, MRM and SIM modes.

1. Introduction

Therapeutic drug monitoring (TDM) of antiepileptic drugs is necessary to optimize patients' clinical outcome by managing their medication regimen with the assistance of measured drug concentrations [1]. Plasma concentration monitoring is widely used for the clinical management of patients with epilepsy receiving a variety of antiepileptic drugs (AEDs) e.g. PHT and LTG [2]. PHT is a hydantoin antiepileptic used to control partial and generalised tonic-clonic seizures. It is also used as part of the emergency treatment of status epilepticus and has been used for the prophylactic control of seizures associated with neurosurgery or severe traumatic injury to the head .the therapeutic range of total plasma-PHT concentrations is usually quoted as 10 to 20 micrograms/mL (40 to 80 micromoles/litre) [3]. LTG is approved for the treatment of partial seizures and seizures associated with Lennox-Gastaut syndrome, a severe form of epilepsy [4]. LTG is also used as first line therapy for childhood absence epilepsy [5]. Several methods have been published for the determination of one or more antiepileptic drugs in biological fluids for TDM or for toxicological purposes. Several high-performance liquid chromatography (HPLC) methods with UV detection for simultanous determination of PHT with certain antiepileptic drugs in biological fluids and drug products are available in the literature [6-11].

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A highly sensitive fluorometric high-performance liquid chromatographic method was developed for the simultaneous determination of PHT and its major metabolites [5-(3-hydroxyphenyl)-5-phenylhydantoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin] [12].

LTG has been determined simultaneously with its impurity [13-14] and antiepileptic drugs [15-16] by using HPLC-UV. Also it was quantified alone by using HPLC-UV [17-18]. A new simultaneous assay was developed to monitor both older and newer AEDs including LTG and PHT using LC/ APCI-MS in selected-ion monitoring (SIM) mode [19]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for determination of PHT were reported and published [20-23]. Hydrophilic interaction chromatography (HILIC) is a liquid chromatography (LC) technique that uses a polar stationary phase (for example, silica or a polar bonded phase) in conjunction with a mobile phase containing an appreciable quantity of water (usually at least 2.5% by volume) combined with a higher proportion of a less polar solvent (often acetonitrile) which lead to enhance sensitivity when used in conjunction with mass spectrometry (MS) because the high organic content of the mobile phase in HILIC allows for efficient spraying and desolvation in electrospray MS [24]. Most AED's are less polar compound and they can be eluted early which lead to decrease the run time of method.

1.1. Objectives

I- Develop a suitable fast, sensitive and selective LC-MS-MS method for the separation and quantification of frequently used antiepileptic drugs LTG and PHT using a suitable HILIC column.

II- Applying the developed method for the determination of these anti-epileptic drugs in human plasma.

2. Experimental

2.1. Materials and chemical

Separation was performed on an Agilent 1200 series system consisting of G1311A Quaternary pump, G1332A Degasser, G1367B HIP-ALS Autosampler, G1316B Thermostatted column compartment and G13115B DAD detector. Mass detection was performed on a model G6410A triple quadrupole(Agilent Technologies, USA). The column used was a HILIC Luna (2×150 I.D) and 3µ particle size (Phenomenex, USA). The mobile phase consisted of acetonitril/ ammonium formate (5mM, pH 3.5) (97.5:2.5, v/v) and was pumped at 0.2ml/min. Samples were introduced to column at an intection volume of 5 µl. Acetonitril, methanol and diethylether were obtained from BDH Laboratory Supplies (poole, UK) and ACROS (USA),ethylacetate was obtained from Winlab laboratory supplies(UK). Anayitical reagents grade ammonium formate, ammonium acetate, potassium carbonate, sodium acetate formic acid and acetic acid were obtained from BDH chemicals (Pool, UK). Water was purified by Millipore system (Milipore, France). The aquoeus mobile phase was filtered through a milipore membrane (HA 0.45 µm). LTG (Fig. 1a) was supplied by aljazeera pharmaceutical industry (Riyadh, saudia arabia). PHT (Fig. 1b) was purchased from Sigma Chemicals, Saint Louis, MO. (THPH) Theophylline (Fig. 1c) was supplied by BDH Laboratory Supplies (poole, UK).

2.2. Preparation of Calibration Standards

Primary stock solutions of LTG, PHT and IS (THPH) (100µg/ml) were separately prepared in acetonitril. The working standards of LTG and PHT (2.25, 3.37, 5.06, 7.59, 11.39 and 17.08 µg/ml) and IS (0.9 µg/ml) were prepared by diluting each primary solution with mobile phase. No internal standard was used with LTG. Human plasma calibration standards and quality control standards containing LTG and PHT were prepared by spiking appropriate amounts of working standard solutions in to drug free human plasma and then serially diluting it with normal blank plasma to attain the desired concentration range. The prepared calibration standards and

quality control standards were prepared in glass ampoules (5ml) and stored at -20 °C until analysis.

2.3. Sample Preparation

To 450µl of plasma was added THPH as internal standard (50µl, 150ng/ml) and 3ml of diethylether in a 5-ml glass ampoule. The samples were vortexed for 1 mint and centrifuged at 5000 *g* for 5 mints. The organic layer was aspirated and transferred to another 5-ml glass ampoule and evaporated under a stream of nitrogen at 40°C. The residue was reconstituted in 450 µL of acetonitril: water (97.5:2.5); vortex-mixed for 50 seconds and 5 µL was introduced into the LC-MS-MS system.

2.4. Instrumental Conditions

Chromatography was performed at ambient tem perature (25°C), at a flow-rate of 0.2 ml/min with acetonitrile/ammonium formate (5mM, pH 3.5) (97.5:2.5, v/v) as mobile phase.The aqueous chromatographic solvents were filtered through a milipore membrane (HA 0.45 µm) before use.

2.5. Mass Spectrometry

Electrospray ionisation was performed in the negative mode for PHT using THPH as IS and positive mode for LTG with the nebulizing gas (nitrogen), gas temperature**,** gas flow and capillary volt set at 50 psi, 350°C, 11 l/min and 5500V, respectively for both analyts. The instrument responses for LTG and PHT were optimised using flow injection analysis. Optimal responses and transition of the protonated (LTG) and deprotonated (PHT) molecular ions are summarized in table 1.

Table 1. MS of anticonvulsant drugs: used and found ions, MS conditions and used transitions

Substance	Molecular weight	Precursor ion	Fragmentor	Collision energy	Dwell time	Used transitions
PHT	252.3	251	130	20	200	251.4 → 102
THPH	180.2	179.1	130	20	200	179.1 → 164.1 179.1 → 122.1
LTG	256	256	120	-	200	256

2.6. Validation

Method validation was performed according to current international regulations on analytical method validation [25-26]. The method was validated by using plasma quality control samples (n=5) at 250, 500 and 1250 ng/ml for LTG and PHT to determine the accuracy and precision of the method. Quality control values were calculated from a standard regression curve, constructed from the ratio of analyte to internal standard peak areas for PHT and peak area for LTG by using six different concentrations. The calibration curve was linear over the concentration range 250–1885 ng/mL for LTG and PHT.

2.7. Matrix effects

Matrix effects were determined by analysing blank biological fluids from six different sources to determine possible interference.

2.8. Recovery

Absolute recovery of the analyte was determined in triplicates at high, medium and low concentrations in normal plasma by extracting drug free plasma samples spiked with LTG and PHT. Recovery was calculated by comparison of the analyte peak-areas (peak ratio for PHT) of the extracted samples with those of the unextracted analyte standards, representing 80 and 90 % recovery respectively.

2.9. Stability

The stability of LTG and PHT stock solutions were evaluated at room temperature for 8 hours and 24 hours and after storage at −20°C for 15 days. Stability was calculated by comparing the pertinent responses obtained from the tested stock solution(s) with the responses of freshly prepared ones and the result are given in Table4. Sufficient number of QC samples at each concentration level (250 and 500 ng/ml) for LTG and PHT were allocated to carry out the short-term stability. Three samples at each level were analyzed for initial concentration determination at zero time. Another QC samples at each level were left on the benchtop at room temperature for 8 hours and 24 hours and then analyzed (3 QC's at each time interval). Stability was calculated by comparing the tested QC samples with those analyzed initially and the result are given in Table4. To perform long-term stability, three QC samples at each concentration level (250 and 500 ng/ml) for LTG and PHT were stored at −20°C for 15 days, and then analyzed. Stability was evaluated by comparing stored samples with those analyzed initially and the result are given in Table 4.

3. Results and discussion

The plasma containing of LTG and PHT were extracted by using different pH modifier. The extracts were found to dirty with low peak area of analytes. The high protein binding PHT and LTG made the extraction with diethylether gave the best results and it was decided to optimize this extraction. The PHT is acidic compound while LTG is basic compound which lead to PHT eluted first. Typical retention times for PHT and its internal standard were 3–3.1 min and 3.55–3.65, for LTG was 4.4–4.5 min. A total run time under 5 min made it possible to analyse more samples per day. Fig.1 a, b and c shows A full scan spectrum (MS2 Scan) spectra for PHT, LTG and THPH with *m*/*z* values of 251, 179.1 and 256 representing molecular ions peaks of the three analytes, respectively. Fig.3. shows representative multiple reaction monitoring (MRM) chromatogram of PHT and THPH and single ion monitoring (SIM) of LTG. As shown the LTG has different mode than PHT and its internal standard. The peak ratio of PHT with its IS (THPH) was linear. The THPH was used to calculate the peak ratio of LTG and the results weren't linear and for this reason the analysis of LTG done without IS. Also, it clear that the results of IS at MRM mode could not be used to calculate the peak ratio of any compound has determined at SIM mode and vice versa. The calibration curve was linear (r=0.997) over the concentration range 250–1885 ng/mL for PHT and 250–1885 ng/mL (r=0.998) for LTG. Fig. 2a shows representative MRM chromatograms of PHT corresponding to the six different calibration standard concentrations (250.0, 375.0, 562.5, 843.7, 1256.6 and 1885.0 ng/ml). Fig. 2b shows representative SIM chromatograms of LTG corresponding to the six different calibration standard concentrations (250.0, 375.0, 562.5, 843.7, 1256.6. and 1885.0 ng/ml). Specificity was evidenced by the lack of interfering peaks in the chromatograms of plasma samples. Figure 4 shows a MRM chromatogram for a blank plasma sample indicating the methods are more specific. Table 2 shows the quality controls data obtained during the validation of the method for PHT and LTG while Table 3 shows the intra-day back calculated quality controls for PHT and LTG. Both intra-and inter-assay CV values ranged from 1.66–8.9% at three QC levels (i.e., 250, 500 and 1250 ng/ml.) for PHT and 0.71-3.7 % at three QC

levels (i.e., 250, 500 and 1250 ng/ml.) for LTG. Results from both intra-and inter-assay CV values (< 9) indicate a valid method. The LODs for PHT and LTG in human plasma were 5.17 ng/ml and 0.13 ng/ml, respectively. The LOQs for both analytes in human plasma were 17.24 ng/ml and 0.46 ng/ml, respectively with using 5µL of human plasma. Data of Stock Solution Stability and Data of Stability in Plasma for PHT and LTG are presented in Table 4.

Table 2: Summary of intra-day quality control results for PHT (n=5) and LTG (n=5)

(A) PHT	QH*	QM	QL
Nominal (ng/ml)	1250	500	250
Mean	213.20	462.9	1241.58
%Nom	85.3	92.6	99.4
RSD (%)	8.9	2.6	5.9
(B) LTG			
	QH	QM	QL
Nominal (ng/ml)	1250	500	250
Mean	1223.16	488	210.54
%Nom	85	97.6	97. 8
RSD (%)	1.49	1.81	3.5

* QH, QM and QL are abbreviations of high, medium and low quality controls, respectively.

Table 3: Summary of back calculated quality control concentrations of PHT and LTG (inter-day variation) showing the repeatability of the method

(A) PHT	QH	QM	QL
Nominal (ng/ml)	1250	500	250
Mean	1260.2	558.17	236
Accuracy (%)	100.8	111.6	94.4
RSD (%)	6.22	2.78	1.66
(B) LTG			
	QH	QM	QL
Nominal (ng/ml)	1250	500	250
Mean	1331.2	502.7	226.8
Accuracy (%)	106.49	100.54	90.73
RSD (%)	3.7	1.56	0.71

Table 4: Summary of stability of PHT, LTG and THPH in stock solution and human plasma.

Data of Stock Solution Stability.			
Drug(n=5)	**8 hrs at RT**	**24 hrs at RT**	**15 Days at −20°C**
PHT			
Precision (%)	4.4	5.04	0.35
Accuracy (%)	107.88	108.35	90.69
LTG			
Precision (%)	3.83	1.43	0.22
Accuracy (%)	108.61	101.9	99.61
THPH			
Precision (%)	1.25	0.098	1.17
Accuracy (%)	103.52	99.15	93.21
Data of Stability in Plasma Samples			
Drug(n=5)	**8 hrs at RT**	**24 hrs at RT**	**15 Days at −20°C**
PHT			
Precision (%)	0.38	1.5	1.42
Accuracy (%)	94.83	95.08	95.41
LTG			
Precision (%)	3.09	0.63	1.84
Accuracy (%)	102.32	102.93	96.35
THPH			
Precision (%)	1.69	2.33	0.355
Accuracy (%)	95.07	91.46	98.05

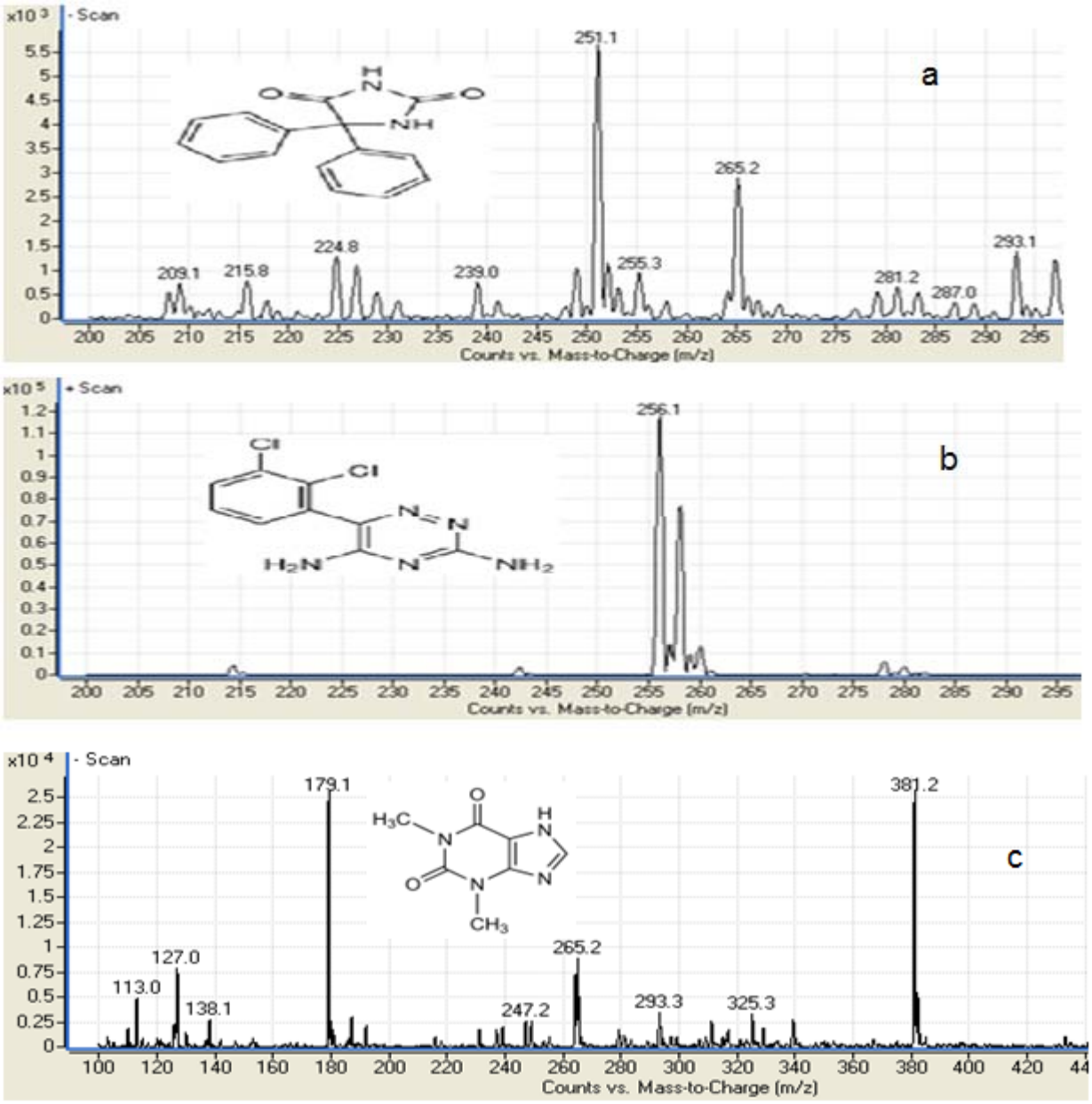

Fig. 1. (a) A MS2 Scan spectrum of a pure solution of PHT in acetonitrile/ammonium formate (5mM, pH 3.5) (97.5:2.5, v/v). Parent [M-1] ion with m/z 251.1 is shown. (b) A MS2 Scan spectrum of a pure solution of LTG in acetonitrile/ammonium formate (5mM, pH 3.5) (97.5:2.5, v/v). Parent [M+1] ion with m/z 256.1 is shown. (c) A MS2 Scan spectrum of a pure solution of THPH in acetonitrile/ammonium formate (5mM, pH 3.5) (97.5:2.5, v/v). Parent [M-1] ion with m/z 179.1 is shown.

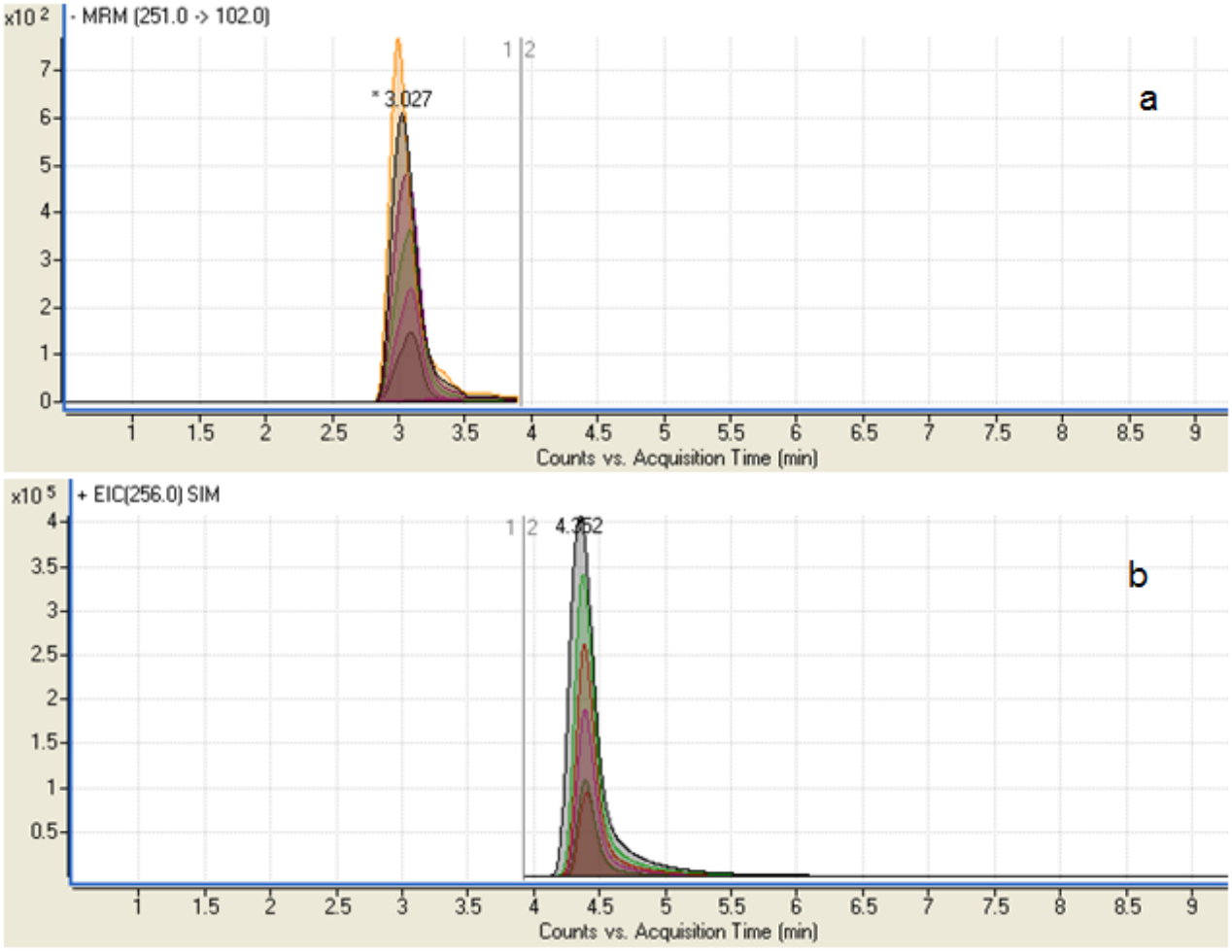

Fig. 2. (a) Peaks of six different concentrations of PHT were used for plasma calibration curve. (b) Peaks of six different concentrations of LTG were used for plasma calibration curve.

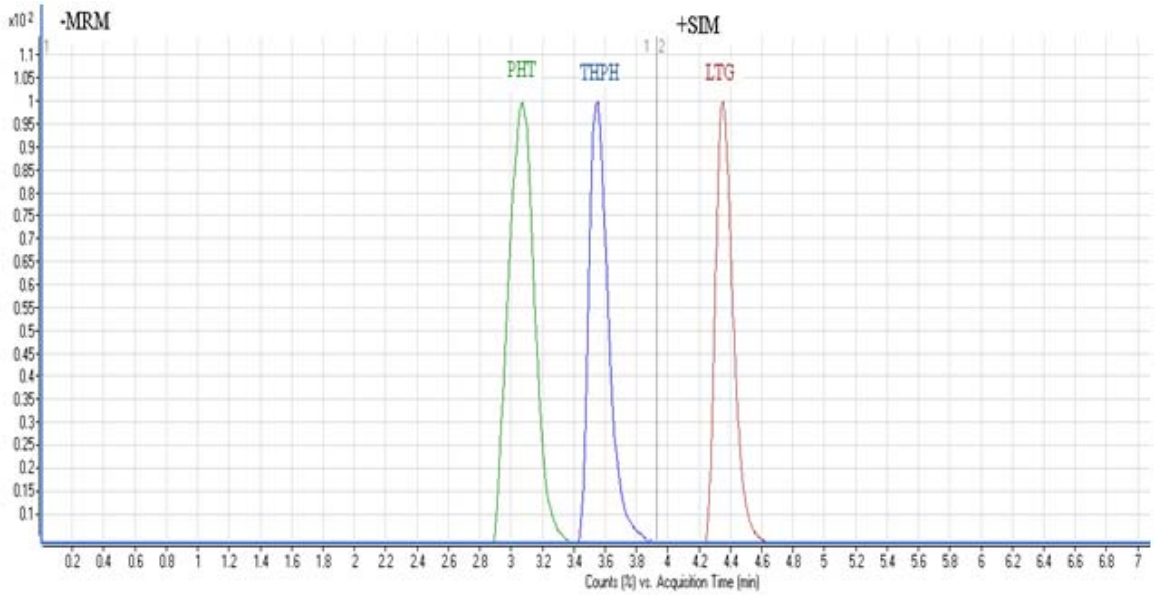

Fig. 3. Representative chromatogram of separated analytes PHT, THPH and LTG

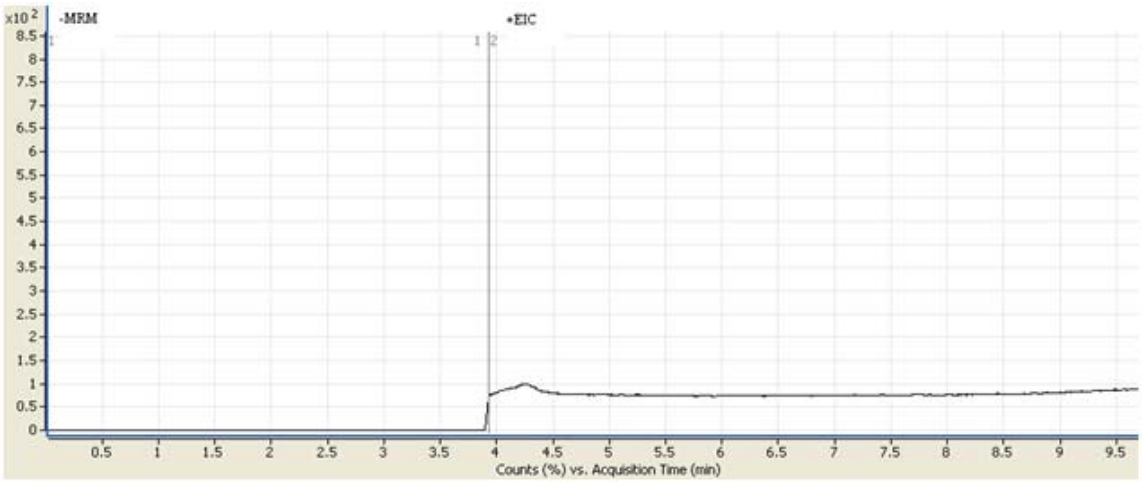

Fig. 4. Chromatogram of blank plasma

4. Conclusion

A highly sensitive, fast and selective method for the detection and quantification of PHT and LTG in human plasma has been developed and validated by using HILIC coupled to triple quadrupole mass spectrometer. To the best of the authors' knowledge, this method is the first reported for simultaneous quantitation of two widely prescribed antiepileptic agents, with one in -MRM mode and the other (LTG) in +SIM mode . A chromatography time of 7 min made it possible to analyze more samples per day.

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