Development of the Method of the Ultra Performance Liquid Chromatography (UPLC) with Photo Diode Array Detector for Determination of 7 Tricyclic Antidepressants' Concentrations in Human Plasma

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ABSTRACT

Objective: Depression is one of the most common human illnesses. Tricyclic antidepressants (TCA) are widely used for the Anti-depressive medication including Amitriptyline, Imipramine, Desipramine, Nortriptyline, Doxepin, Trimipramine and Clomipramine. The adverse effects have become major medical problems with severe morbidity and mortality from their cardiovascular and neurological toxicity. Several studies have reported the relationship between their toxic effects and elevated plasma concentration of TCAs. Therefore, therapeutic drug monitoring (TDM) of these drugs might be useful. This study aimed to develop and validate the method of ultra performance liquid chromatography with photodiode array (UPLC/PDA) for determination of 7 TCAs' concentrations in human plasma.

Methods: Chromatographic separation was carried out by the ACQUITY UPLC™ BEH Shield RP, 1.7 μm (100 x 2.1 mm.I.D.) and used Acetronitrile/Phosphate Buffer Solution (pH 8) with gradient program as a mobile phase. The ACQUITY UPLC® Photodiode Array (PDA) Detector was performed at wavelengths between 200-380 nm and quantification was done at 215 nm. The Solid Phase Extraction (SPE), was used for extraction. The method was developed and fully validated according to USFDA guideline.

Results: This method has an excellent separation result for 7 TCAs' concentrations in 5 minutes. The limit of quantification (LOQ) was 20 ng/mL for all TCAs. This method was fully validated in terms of selectivity, accuracy, precision, linearity (r^2>0.9975) and stability. The recoveries of extraction ranged from 56.11-76.37% with repeatability.

Conclusion: A rapid, sensitive, accurate and reproducible UPLC/PDA method for quantification of TCAs level in human plasma were developed and validated, suggesting that the developed methods are useful for the future routine analysis.

Keywords: UPLC/PDA, tricyclic antidepressant, therapeutic drug monitoring

INTRODUCTION

Tricyclic antidepressants (TCAs) are heterocyclic compounds widely used for psychiatric medication or mood disorder including clinical depression[1], attention-deficit hyperactivity disorder[2] and chronic pain.[3,4] The most common TCAs include Amitriptyline, Imipramine, Desipramine, Nortriptyline due to their high efficiency with low cost. Moreover, Doxepin, Trimipramine and Clomipramine are also widely prescribed in Asia. Due to the widespread use of these drugs, the unattended poisoning from their side effects has become a major medical problem.[5,6]

The pharmacokinetic studies of TCAs show high inter-individual variability from the heterogeneity of the hepatic metabolism and elimination.[7,8] Furthermore, the overdose of TCAs associated with severe morbidity and mortality is well documented due to their cardiovascular[9] and neurological toxicity.[10] Dose optimization is often

required whilst patients'are undergoing treatment, therefore the therapeutic drug monitoring may be needed.[11-13] A numbers of analytical methods have been reported to determine TCAs in biological fluids for TDM or in toxicology purposes, and these include Spectrophotometry[14], High performance thin layer chromatography (HPTLC)[15], High performance liquid chromatography (HPLC)[15-21] and Liquid chromatography tandem mass spectrometry(LC-MS/MS).[22-24] However, highly specific and highly sensitive techniques with short runtime are the requirement of TDM. Moreover, it should cover the main TCAs prescribed. UPLC is a novel development in the liquid chromatography method. It represents a significant decrease in separation time without loss of its efficiency and resolution. In this study, we aimed to develop a UPLC-PDA method and fully validate it for determination of the seven most frequently prescribed TCAs in Asia. We expect that our method will be useful for monitoring these drugs' level in clinical practice in the near future.

MATERIALS AND METHODS

1. Chemicals

Amitriptyline, Imipramine, Desipramine, Nortriptyline Doxepin, Trimipramine, Clomipramine and Carbamazepine were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany). The HPLC grade acetonitrile and methanol (MeOH) were purchased from Labscan Ltd. (Bangkok, Thailand). Milli-Q water from water purification system, Millipore Corporation (Massachusetts, USA) was used. Other chemicals were of analytical grade. Drug-free human plasma was from the Department of Transfusion Medicine, Siriraj Hospital. The protocol was approved by Siriraj Institution Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2. Instrumentations

Acquity[TM] Ultra Performance Liquid Chromatography from Waters Co., Ltd. (USA) was used for the separation module. Chromatographic separation was carried out on the ACQUITY UPLC™ BEH Shield RP, 1.7 µm (100 x 2.1 mm.i.d.) from Waters Co. Ltd. (USA). To get the optimum result, mobile phase with a flow rate of 0.5 mL/min and column temperature at 40ºC were used. The gradient programme for the mobile phase was optimized using Acetronitrile/Phosphate Buffer Solution (pH 8) 35/65 (v/v) at initial, 55/45 (v/v) at 3 min and 35/65 (v/v) at 5 min. The ACQUITY UPLC® Photodiode Array (PDA) Detector from Waters Co. Ltd. (USA) was used at wavelengths between 200-380 nm and quantitation was done at 215 nm. The Empower 2 software was used for data management.

3. Standard stock solutions

Standard stock solutions of Amitriptyline, Imipramine, Desipramine, Nortriptyline, Doxepin, Trimipramine, Protriptyline, Clomipramine and Carbamazepine were prepared with MeOH/Milli-Q (50/50, v/v). Carbamazepine was used as an internal standard (IS). The dilution of stock solution to concentration, which ranged from 20-1,000 ng/mL, was used as working solution.

The Quality Control (QC) samples were prepared at 20, 60, 450, and 950 ng/mL. Standard solutions were stored at -20 degree Celsius until use.

4. Sample preparations

The Solid Phase Extraction (SPE), Oasis Mixed-mode cation exchange sorbent for bases (MCX) 30 mg 1 mL was used as a mixed mode reversed-phase/cation-exchange cartridge for sample preparation. The SPE MCX cartridges were initially conditioned with 1 mL of methanol and equilibrated with 1 mL of Milli-Q water before use. A 50 µL of IS, Carbamazepine (50 ng/mL) was added into 0.8 mL of plasma samples. Then, the plasma samples were aspirated into the wetted preconditioned SPE MCX cartridges. Afterwards, the plasma components were washed with two steps of the wash solvent (1 mL of 2% CH_3COOH and 1 mL of MeOH 1 mL). TCAs were subsequently eluted from the dried columns using 0.5 mL of an eluting solution (5% NH_4OH in MeOH). A 400-µL elute was then diluted with 200 µL of 0.05% Trifluoroacetic acid (TFA) before injection into the UPLC system.

5. Bioanalytical method validation

The developed method was fully validated according to the USFDA guidline.[25]

5.1 Selectivity and sensitivity

The selectivity was examined using six sources of free drug plasma which were extracted and analyzed by the developed method. The result should not have any interfering peak of TCAs. The sensitivity at the limit of quantification (LOQ) was also examined by dilution of standard compounds in plasma which were extracted and then quantified for the lowest detectable concentration.

5.2 Accuracy and precision

Accuracy and precision were examined by six replicate analyses of plasma spiked with four different concentrations (LOQ, 60, 450 and 950 ng/mL) for three separate days. The percentage of relative error (%RE), indicating accuracy, was calculated as the measured concentration divided by the spiked concentration. The percentage of coefficient of variation (%CV), indicating precision, was obtained from the ratios of standard deviation (SD) to the mean of the measured concentration. Both %RE and %CV should be within ± 20% at LOQ and 15% at other concentrations.

5.3 Linearity and calibration curve

A calibration curve was represented by a linear regression model, $y=mx+b$ and weighting by $1/x$, where y is the ratio of peak area of analyze to the peak area of IS, x is the concentration at different levels including 20, 150, 300, 500, 750 and 1,000 ng/mL. All calibration ranges yielded linear relationships with coefficients of determination (r2) and their value must exceed 0.995.

5.4 Recovery of extraction

The recovery method was performed by comparing peak areas of the extracted samples at three different concentrations (60, 450 and 950 ng/mL) with peak areas of non-extracted standard solutions at the same concentrations. The percentage of absolute recovery (%RV) was the ratio of the measured extracted peak area to the non-extracted peak area.

5.5 Stability

The stability of analysis was performed by three replicate analyses of plasma spiked with three different concentrations (60, 450 and 950 ng/mL) under various conditions with freshly prepared samples. The first condition was freeze and thaw in which plasma samples were frozen at -20°C and thawed at room temperature for three cycles before analysis. For short term stability tests, the plasma samples were stored at room temperature for 6 hours before analysis. Post-preparative stability tests were done by placing the vials of plasma samples in the autosampler at 8°C for 10 hours before analysis. Lastly, for long term stability, plasma samples were frozen at -20°C for 1 month before analysis. The acceptable percentage of variation in each condition must be within ±15%.

RESULTS

To get the optimum results, the gradient UPLC conditions were optimized and the chromatographic separation was described in the materials and methods section. This method had an excellent separation for 7 TCAs' plasma levels and internal standard which were achieved within 5 min. The appropriate retention time when spiked with plasma were 1.266 min for IS, 1.477 min for Desipramine, 1.683 min for Nortriptylone, 2.032 min for Doxepin, 2.265 min for Imipramine, 2.777 min for Amipramine, 3.352 min for Clomipramine and 3.623 min for Trimipramine as shown in Fig 1. Representative chromatograms are shown with PDA spectrum in Fig 2. The capacity factor (k') was in the range 1.109-5.038 while the resolution factor was great in the range 4.202-9.239 as shown in Table 1.

No interference of matrix compound was observed in selectivity testing as shown in Fig 1. The limit of quantification (LOQ) was demonstrated at 20 ng/mL for all
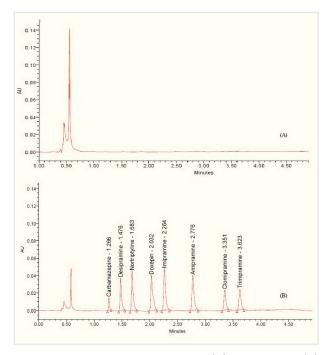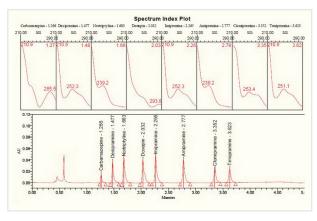

Fig 2. The PDA spectrum of TCAs.

which used 500 µL of plasma sample. This method had good accuracy, the %RV was found to be in the range of 95.21-110.63% for all TCAs. High precision was shown in both inter-day and intra-day testing variation, and %CV was found to be in the range of 2.46-14.57%. The accuracy and precision were acceptable as summarized in Table 2. This method had a good linearity with the coefficient of determination (r^2) more than 0.997 as summarized in Table 3. All were within the acceptable range ($r^2 > 0.995$). The extraction efficiency (%RE) was found to be in the range of 56.11-76.37% as shown in Table 3. This study show that the plasma samples containing TCAs were stable in various tested condition as demonstrated in Table 4. The percentage of variation in each condition was within the acceptable range.

DISCUSSION

The solid phase extraction (elution step) was optimized in order to obtain a simple procedure for routine analysis than previously published procedures due to no evaporating step. The applied chromatography condition permitted a good separation of each TCA in different concentrations and no interference of TCAs was observed during this analysis. The recoveries of extraction were low but repeatable. The relative retention times which presented in the plasma samples were confirmed by injection of each standard and by comparison of their PDA spectrum between 200-380 nm.

Fig 1. The chromatograms of extracted (A) blank plasma, (B) plasma spiked with 150 ng/mL of TCAs and IS.

TABLE 1. The capacity factor (k') and resolution of TCAs chromatogram

Compound	Retention time	Capacity factor	Resolutions
Carbamazepine	1.266	1.109	-
Desipramine	1.476	1.460	5.874
Nortriptyline	1.683	1.804	4.641
Doxepin	2.032	2.386	6.870
Imipramine	2.264	2.774	4.202
Amipramine	2.776	3.627	8.658
Clomipramine	3.351	4.585	9.239
Trimipramine	3.623	5.038	4.271

TABLE 2. The precision and accuracy (inter-day and intra-day).

	Expected concentration (ng/mL)	Precision (%CV)		Accuracy (%RV)
		Within day (n=6)	Between day (n=18)	
Amipramine	20	2.99	8.92	110.63
	60	4.69	6.55	109.27
	450	4.09	6.10	105.74
	950	4.71	7.98	101.02
Clomipramine	20	9.31	12.05	99.80
	60	8.66	8.51	98.31
	450	8.48	7.50	98.96
	950	6.85	7.70	98.72
Desipramine	20	8.48	14.57	110.48
	60	8.17	7.05	101.66
	450	6.68	8.27	102.47
	950	6.47	6.85	103.25
Doxepin	20	7.44	11.16	103.67
	60	2.54	7.99	103.97
	450	8.25	9.80	100.71
	950	6.60	5.77	95.21
Imipramine	20	5.92	12.13	98.49
	60	5.84	7.47	105.18
	450	6.34	5.96	97.40
	950	6.42	6.37	94.66
Nortriptyline	20	6.42	10.67	103.23
	60	4.90	6.18	105.01
	450	6.60	7.01	103.97
	950	8.48	6.95	101.87
Trimipramine	20	6.38	12.15	96.38
	60	5.90	5.62	102.07
	450	9.04	7.85	97.78
	950	2.46	6.80	95.13

n = number of replicate

CONCLUSION

The UPLC/PDA method for detection of 7 TCAs in human plasma was developed and fully validated according to USFDA guideline. This method is suitable for routine analysis in the near future with a simple and rapid assay for the quantitative determination.

TABLE 3. The linearity and recovery of TCAs

Compound	Concentration range (ng/mL)	Coefficient of determination (r^2)	Recovery of extraction (% RE)	Therapeutic range (ng/mL)
Amipramine	20-1000	0.9967	59.67	120-250[a]
Clomipramine	20-1000	0.9960	68.16	160-400
Desipramine	20-1000	0.9987	76.37	115-250
Doxepin	20-1000	0.9975	56.11	150 -250
Imipramine	20-1000	0.9988	74.25	180-350[a]
Nortriptyline	20-1000	0.9966	71.50	50-150
Trimipramine	20-1000	0.9985	71.13	150-350

[a] Drug plus metabolite.

TABLE 4. The stability of TCAs.

Compound	Variation (%)			
	Freeze and thaw	Short term	Long term	Post-preparative
Amipramine	3.64 - 7.72	3.19 - 10.45	5.06 – 9.39	1.35 - 5.45
Clomipramine	1.22 - 10.91	1.60 - 7.51	9.15 - 13.42	0.74 - 11.78
Desipramine	0.14 - 4.21	1.75 - 7.48	0.33 - 7.80	0.31 - 1.91
Doxepin	3.59 - 6.54	0.23 - 4.92	5.04 - 8.04	2.66 - 8.29
Imipramine	1.51 - 5.06	0.40 - 4.86	2.22 - 6.71	0.70 - 7.96
Nortriptyline	1.05 - 2.74	0.08 - 4.69	4.44 - 5.26	0.04 - 3.01
Trimipramine	5.60 - 7.11	1.31 - 4.86	1.70 - 4.47	1.57 - 3.91

n = number of replicate

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