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Short communication

Validated LC–MS (ESI) assay for the simultaneous determination of amitriptyline and its metabolite nortriptyline in rat plasma: Application to a pharmacokinetic comparison

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ABSTRACT

A rapid, sensitive and specific method based on high performance liquid chromatography with electrospray ionization mass spectrometry (HPLC–MS/ESI) has been developed for the simultaneous determination of amitriptyline and nortriptyline in rat plasma. Sample preparation involved liquid–liquid extraction with methyl t-butyl ether after alkalified with 0.5 mol/l NaOH. Chromatographic separation was performed on a XB-C4 column (4.6 mm × 250 mm, 5 μm, Welch Materials) with a mobile phase consisting of 10 mM ammonium acetate (0.6‰ formic acid)–acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min. Calibration curves were linear within the ranges of 10–3200 ng/ml for amitriptyline and 10–1000 ng/ml for nortriptyline. This method was successfully applied to the pharmacokinetic study in rats after intravenous injection of amitriptyline hydrochloride.

1. Introduction

As a typical tricyclic antidepressant (TCA), amitriptyline has been frequently used for the treatment of major depression since its introduction in the 1960s. It generates a definite pharmacodynamic effect, mainly by blocking the pre-synaptic uptake of amines (norepinephrine, dopamine and serotonin). Metabolism of amitriptyline involves hepatic microsomal enzyme (mainly CYP2C19 and CYP3A4) that causes demethylation of the aliphatic side chain, which generates pharmacologically active metabolite nortriptyline [1] (Fig. 1). The drug has a relatively narrow therapeutic index, so overdosing may lead to severe poisoning, including cardiovascular, respiratory and central nervous system toxicity. TCA overdose is responsible for a significant proportion of severely poisoned patients in hospital. The effort of seeking for an effective treatment is ongoing.

Recently, some studies [2–4] revealed that amitriptyline and nortriptyline are substrates of a plasma membrane phosphoglycoprotein, P-glycoprotein (P-gp). In addition, a number of reports [5–7] noticed that dexamethasone (DEX) is an inductor of P-gp and CYP3A4. Thus, we propose a tentative idea that DEX might speed up the elimination of poisoning by strengthening the P-gp meditated drug efflux and inducing the hepatic microsomal enzyme. Verapamil (VER), as a competitive inhibitor of P-gp, can be used to confirm the importance of this transporter in poison excretion [8]. In order to prove our theory, we made a comparison of the kinetics of amitriptyline and nortriptyline between rats under different drug pretreatments.

To date, various assays of amitriptyline and its metabolites in biological samples have been reported. These are mainly based on reversed-phase separation followed by UV [9–11] or particle beam mass spectrometric determination [12]. However, the sensitivity of these methods appears too low: leading to the need for a large sample volume. Kollroser and Schober [13] described a liquid chromatography with tandem mass spectrometry method that achieves better sensitivity. However, it used 1 ml plasma aliquot to reach the low quantification limit. In addition, the on-line sample extraction still needs manual sample preparation and extra instrumentation.

In order to facilitate the present study in rat where large sample volumes are not available, we developed a simple and sensitive method for extraction and determination of amitriptyline and nortriptyline in plasma.

2. Experimental

2.1. Equipments and reagents

A system of HPLC–MS (Waters 2690, USA) with a micromass ZQ mass spectrometer (Wythenshawe, Manchester, UK) equipped

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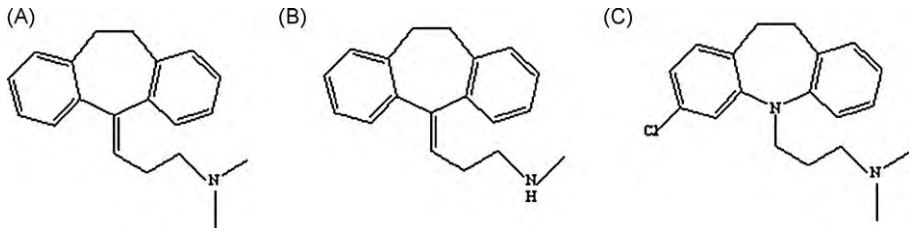

Fig. 1. Structures of amitriptyline (A), nortriptyline (B) and internal standard clomipramine (C).

with an electrospray ionization (ESI) ion source was used. COMPAQ Deskpro Workstation and MassLynxTM 3.5 software were utilized.

Nortriptyline hydrochloride (>98%) and RS-verapamil hydrochloride (>99%) were purchased from Sigma Chemical Co. (St. Louis, USA). Dexamethasone was purchased from Tianjin JinJin Pharmaceutical Co., Ltd. (Tianjin, P.R. China). Amitriptyline hydrochloride (>99%) and clomipramine hydrochloride (>99%) were generously donated by Hunan Dongting Pharmaceutical Co., Ltd. (Hunan, P.R. China). Acetonitrile, methanol, methyl t-butyl ether and formic acid (HPLC grade) were purchased from Tedia Company Inc. (Fair Field, USA). Other AR grade reagents (acetic acid, sodium hydroxide) were obtained from the Chemical Reagent Factory of Hunan (Hunan, P.R. China).

2.2. Standard solutions

The primary stock solutions of amitriptyline (160.0 μg/ml), nortriptyline (101.7 μg/ml) and the internal standard (I.S.) clomipramine (125.8 μg/ml) were prepared in methanol. Working solutions were obtained by diluting the stock solutions with double distilled water. All solutions were kept away from light and stored at 4 °C.

Calibration standards were prepared by adding appropriate volumes of working solutions into drug-free rat plasma. The final concentrations were 10, 25, 50, 100, 250, 500, 1000 ng/ml for nortriptyline and 10, 30, 100, 300, 600, 1200, 3200 ng/ml for amitriptyline. Quality control (QC) samples run in each assay were prepared in the same way. Final amitriptyline and nortriptyline concentrations were 10, 300, 3200 ng/ml and 10, 100, 1000 ng/ml, respectively.

2.3. Chromatographic and MS/ESI detection conditions

The analytes were separated on a Welch C4 (4.6 mm × 250 mm, 5 μm, U.S.) column with column temperature of 40 °C. Isocratic elution employed a mobile phase of buffer (formic acid: 0.6‰, NH_4Ac: 10 mM)–acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min. The postcolumn splitting ratio was 3:1. Ionization of the analytes was obtained by electrospray in the positive ion mode (ESI+). The optimized working parameters were: capillary voltage, 3.00 kV; extractor voltage, 3.00 V; cone voltage, 28.00 V for amitriptyline, 23.00 V for nortriptyline, 23.00 V for clomipramine (I.S.); source temperature, 120 °C; desolvation temperature, 300 °C; cone gas flow, 100 l/h; desolvation gas flow, 300 l/h. Selected ion recording (SIR) mode was used for quantitation by the protonated molecular ions of each analyte.

2.4. Sample preparation

A 100 μl aliquot of rat plasma was mixed with 20 μl I.S. solution and 25 μl 0.5 M NaOH before being extracted with 2 ml methyl t-butyl ether by vortex shaking for 90 s. Following centrifugation at 3000 rpm for 5 min, the organic layer was transferred to another tube and evaporated at 40 °C under a gentle stream of nitrogen. The dry residue was then reconstituted with 100 μl mobile phase and vortex-mixed for 20 s. A 20 μl solution was injected into the HPLC–MS/ESI.

2.5. Validation of the method

The method validation assays were carried out according to the currently accepted U.S. Food and Drug Administration (FDA) bioanalytical method validation guidance [14]. Specificity (the absence of interferences from endogenous components in the biological matrix or exogenous components from the isolation procedure) was assessed by extracting control blank plasma samples in each validation run. The lack of interfering peaks at the same analyte retention time was considered as the acceptable selectivity. Calibration curves were generated from seven standards by performing a weighted least-squares linear regression ($1/x$) of the peak area ratios of the amitriptyline or nortriptyline to the I.S., versus the respective standard concentration. Intra- and inter-day precision (R.S.D.) and accuracy (R.E.) assays were carried out five times, using three different concentrations on the same day and over 5 different days. The extraction recoveries were determined at three concentration levels by comparing the analytes peak areas obtained from the QC samples ($n = 5$) after extraction to those of post-extraction blank matrix extracts (direct extract from blank plasma) spiked at the corresponding concentrations. Matrix effects were evaluated by comparing the peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions. To assess the stability of analytes in plasma samples, five replicates of QC samples were subjected to short-term (12 h) room temperature, two freeze/thaw (−20 to 25 °C) cycles and long-term (30 days, −70 °C) stability tests.

2.6. Drug administration and sample collection

Animal experiments were carried out according to institutional guidelines for the care and the use of laboratory animals, and approved by the Animal Ethics Committee of Central South University. Fifteen male Wistar rats (250–300 g) were obtained from the laboratory animal center of the Second Xiangya Hospital. Before starting the experiments, animals were kept under standard laboratory conditions (12/12 h light/darkness, 22 ± 2 °C room temperature, 50–60% humidity) for at least 1 week. Rats were then divided into three groups of five animals each. The three groups were treated as follows. (i) The control group ($n = 5$) animals were injected (i.p.) daily with corn oil for 4 days. (ii) The DEX group ($n = 5$) animals were similarly treated daily for 4 days with a corn oil solution of DEX (25 mg/kg). (iii) The VER group ($n = 5$) animals were treated with corn oil as the control group and injected verapamil (8 mg/kg, i.p.) 1 h before administration of amitriptyline on the fifth day. Following an overnight fast, amitriptyline hydrochloride was given to all rats by intravenous injection (5 mg/kg). Heparinized venous blood samples (300 μl each) were collected according to designated time intervals via the post-orbital venous plexus veins, which is at 5, 10, 30, 60, 120, 200, 300 and 400 min after drug administration. Plasma (100 μl) was immediately separated by

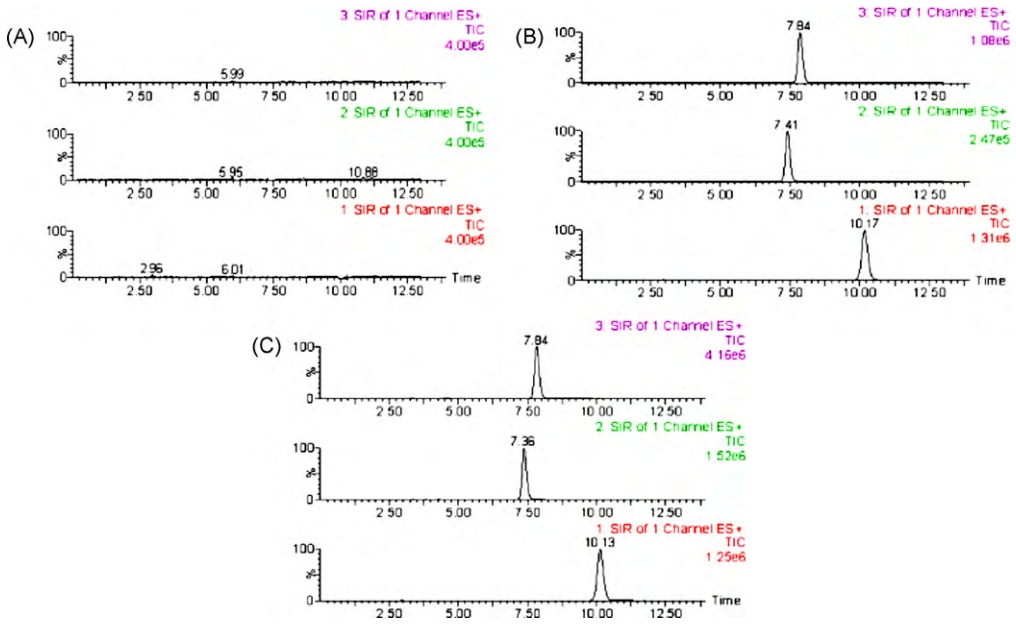

Fig. 2. Typical MRM chromatograms of clomipramine (channel 1) I.S., nortriptyline (channel 2) and amitriptyline (channel 3). (A) Chromatograms of a blank plasma sample; (B) plasma sample spiked with amitriptyline 300 ng/ml, nortriptyline 100 ng/ml and I.S.; (C) a rat plasma sample 10 min after intravenous injection of 5 mg/kg amitriptyline.

centrifugation at 5000 rpm for 10 min, then transferred to suitably labeled tubes and stored at −70 °C until assay.

3. Results and discussions

3.1. Assay development

The HPLC–MS/ESI in the SIR mode provided a highly selective method for the determination of amitriptyline, nortriptyline and clomipramine (I.S.). The analytes were easily protonated and generated positive product ions. They were identified at *m/z* 278 for $[\text{amitriptyline}+\text{H}]^+$, 264 for $[\text{nortriptyline}+\text{H}]^+$ and 315 for $[\text{I.S.}+\text{H}]^+$. The retention times were approximately 7.8, 7.4 and 10.2 min, respectively. Typical chromatograms resulting from the analysis of various plasma samples are shown in Fig. 2. No endogenous substance or chemical components were observed to interfere with the drugs and internal standard over the concentration range.

In our assessment of different mobile phases, analytes on the C18 column will only be washed off when the concentration of ammonium acetate buffer is high enough (≥50 mM). However, in order to avoid signal interference with the mass detector, a low concentration buffer is preferable. Facing this defect, we chose the reverse phase C4 column instead of the more frequently used C18 column. As the column hydrophobicity became weaker, the basic analytes appear in better peak shape and can be washed off under relatively low buffer concentrations (10 mM).

We compared three extraction solvents (acetic ether, ether and methyl t-butyl ether) and finally chose methyl t-butyl ether as the solvent for liquid–liquid extraction, because it has an appropriate recovery (methyl t-butyl ether 83%, acetic ether 45%, and ether 60%), good evaporability and only needed 2 ml. The time of extraction and centrifugation was also shortened.

3.2. Assay validation

Calibration curves were linear over the concentration range 10–3200 ng/ml for amitriptyline, 10–1000 ng/ml for nortriptyline. The regression equations for amitriptyline and nortriptyline were $y = 0.0019c + 0.0112$ ($r = 0.9991$) and $y = 0.0016c + 0.0019$ ($r = 0.9997$), respectively. The limit of quantification (LOQ) validated was 10 ng/ml (S/N > 10) defined as the lowest concentration at which the R.S.D. was below 20%.

Table 1 summarizes accuracy and precision for the analytes in rat plasma based on analysis of QC samples. The extraction

Table 1
Accuracy, precision, and stability of amitriptyline and nortriptyline in rat plasma.

	Theoretical concentration (ng/ml)					
	Amitriptyline			Nortriptyline		
	10	300	3200	10	100	1000
Accuracy and precision						
Intra-day precision ($n = 5$)						
Mean ± S.D. (ng/ml)	9.9 ± 0.7	317.5 ± 14.4	3253.7 ± 91.3	10.1 ± 0.6	97.2 ± 4.8	983.9 ± 23.6
Accuracy (R.E. %)	−1.1	5.8	1.7	0.8	−2.8	−1.6
R.S.D. (%)	6.9	4.5	2.8	5.5	5.0	2.4
Inter-day precision ($n = 5$)						
Mean ± S.D. (ng/ml)	9.8 ± 1.1	334.8 ± 19.9	3367.0 ± 168.3	9.3 ± 0.8	92.6 ± 5.2	963.7 ± 40.2
Accuracy (R.E. %)	−1.7	11.6	5.5	−7.0	−7.4	−3.6
R.S.D. (%)	11.6	6.0	5.0	8.8	5.6	4.2
Stability of samples ($n = 5$)						
Short-term (%)	93.8 ± 6.1	104.8 ± 6.1	101.8 ± 4.4	96.4 ± 5.8	102.3 ± 4.3	101.4 ± 2.2
Long-term (%)	94.2 ± 8.5	109.1 ± 6.9	111.4 ± 4.9	93.8 ± 5.7	101.1 ± 6.9	99.4 ± 3.4
Freeze/thaw (%)	97.2 ± 10.6	111.0 ± 6.9	105.9 ± 5.4	93.6 ± 8.5	96.9 ± 8.4	94.7 ± 7.0

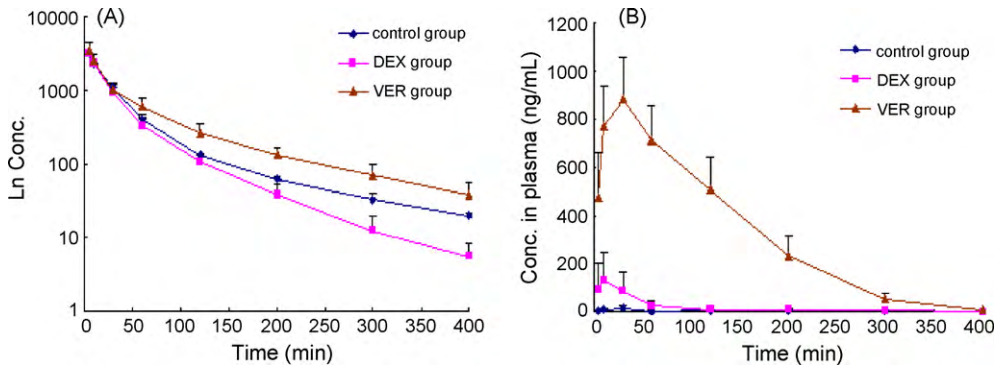

Fig. 3. Mean plasma concentration–time profiles of amitriptyline (A) and nortriptyline (B) after intravenous injection of 5 mg/kg amitriptyline, each point and bar represents the mean ± S.D. ($n = 5$). (A) *Y*-axis: ln conc. (B) *Y*-axis: conc.

Table 2
Pharmacokinetic parameters of amitriptyline and nortriptyline after a single i.v. administration of amitriptyline (5 mg/kg) in control, DEX and VER groups.

Drug	Parameter	Control	DEX	VER
Amitriptyline	$AUC_{0-\infty}$ (μg min/ml)	101.9 ± 28.1	87.1 ± 24.0	135.7 ± 36.5
	MRT (min)	95.2 ± 9.4[b]	43.4 ± 4.8[a,c]	96.9 ± 9.3[b]
	CL_{tot} (ml/min)	32.3 ± 2.1[b]	63.3 ± 3.6[a,c]	33.0 ± 2.3[b]
	Vd_{ss} (ml)	3059.2 ± 158.5	2989.5 ± 181.8	3189.4 ± 154.0
Nortriptyline	$AUC_{0-\infty}$ (μg min/ml)	1.8 ± 1.0[c]	6.9 ± 5.4[c]	127.3 ± 25.4[a,b]
	T_{max} (min)	22.0 ± 10.0	16.0 ± 9.0	24.0 ± 8.0
	C_{max} (ng/ml)	13.5 ± 13.2[c]	134.2 ± 116.5[c]	880.7 ± 175.2[a,b]

[a] Significantly different from control rats at $P < 0.05$.
[b] Significantly different from DEX-treated rats at $P < 0.05$.
[c] Significantly different from VER-treated rats at $P < 0.05$.

recoveries of amitriptyline and nortriptyline were in the range of 79.6–88.9% and 79.4–90.5%, respectively. No matrix components in plasma caused significant changes in the MS response of analytes. Studies of matrix effects of two analytes at QC concentrations gave concentrations within ±10% of nominal values (91.4–104.3% for amitriptyline and 93.4–102.5% for nortriptyline).

Stability quality control plasma samples were found to be stable in the plasma when placed in the short-term (12 h) room temperature, two freeze/thaw (−20 to 25 °C) cycles and stored at −70 °C for 30 days (Table 1).

3.3. Pharmacokinetic comparison

A plot of the mean plasma amitriptyline and nortriptyline concentrations is presented in Fig. 3. The pharmacokinetic parameters were estimated according to model-independent moment analysis and expressed as means ± S.D. of five rats (Table 2). Statistical comparison between groups was estimated using one-factor ANOVA followed by SNK test.

By comparing the pharmacokinetic parameters of amitriptyline, the DEX-treated group evidenced a significant decrease in the values of MRT, whereas the values of CL_{tot} were significantly increased. But the values of $AUC_{0-\infty}$ and Vd_{ss} showed no significant difference between the three groups.

On the other hand, the $AUC_{0-\infty}$ and C_{max} of nortriptyline of the control group were 1.80 ± 1.0 (mean ± S.D.; μg min/ml) and 13.54 ± 13.2 (mean ± S.D.; ng/ml). Rats treated with VER evidenced a significant increase, up to 127.31 ± 25.4 and 880.72 ± 175.15 ($P < 0.05$). Although the DEX-treated group manifests higher values of $AUC_{0-\infty}$ and C_{max}, they showed no significant difference from the control group.

As we expected, the pre-administration of DEX increased the elimination of amitriptyline in rats. However, the P-gp inhibitor VER did not change its disposition kinetics, which suggests that the increase of CL_{tot} mainly results from the induction of hepatic microsomal enzyme. On the contrary, the increase of $AUC_{0-\infty}$ and C_{max} of nortriptyline after VER administration suggests that P-gp plays a more important role in the disposition of nortriptyline.

4. Conclusion

As demonstrated in this assay, our method is sensitive, accurate and specific. In practical applications, this method meets the request of the present pharmacokinetic comparison and is suitable for the analysis of samples in batches.

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