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Determination of amitriptyline and nortriptyline in human liver microsomes with reversed-phase HPLC *in vitro*[1]

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KEY WORDS amitriptyline; nortriptyline; high pressure liquid chromatography; liver microsomes

AIM: To develop a method for simultaneous determinations of amitriptyline (Ami) and its metabolite nortriptyline (Nor) in human liver microsomes. **METHODS**: An incubation buffer containing microsomes, NADPH-generating system, and Ami, after termination of enzyme reaction and desipramine (Des) as internal standard (IS), was extracted with diethy ether and separated on a reversed-phase ODS column. Detection was achieved at 242 nm by ultraviolet detector. **RESULTS**: No potential interfering peaks were found. Ami and Nor gave rapid elution and baseline resolution. The linear curves of both analyses ranged 0.02 - 10 nmol and the limit of detection was 0.01 nmol. The recovery (94 % - 101 %) had good precision with relative *s* of < 8.3 %. **CONCLUSION**: This method is rapid, sensitive, and simple for studying the metabolism of Ami and Nor.

In recent years, the use of human liver microsomal preparations for studying the *in vitro* metabolism of drug has increased greatly[1]. Due to specific characteristics of *in vitro* studies of drug metabolism with liver microsomal preparations, the method for determining a drug and its metabolite(s) in plasma and urine of *in vivo* studies can not be applied to *in vitro* studies without modifications. For precise analysis of amtriptyline (Ami) and nortriptyline (Nor) in human plasma and/or urine, HPLC with ultraviolet (UV) detector was the preferred method in most laboratories[2-6]. But their utility in *in vitro* studies of drug metabolism is somewhat hampered by either narrow rang of linearity (20 - 300 mg · L^{-1})[2-5], time-consuming extraction[4,5], or requirement of a column switching system[3,6]. In addition, the HPLC method reported previously for only determining Nor in human liver microsomal preparations[7,8] was unsatisfactory because of its relative low sensitivity (the limit of detection was 0.05 nmol Nor). Accordingly, a rapid, sensitive, simple, and reproducible analytic method is required for the purpose of investigating metabolism of Ami or Nor *in vitro*. This paper described a reversed-phase HPLC method for simultaneous quantitation of Ami and Nor in human liver microsomes.

MATERIALS AND METHODS

Chemicals Ami, Nor, desipramime (Des), NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma. Acetonitrile (Linhan Chemical Factory, Zhejiang, China) of HPLC grade and doubly distilled water were required for HPLC with UV detector. All other chemicals were of AR grade.

Standard solutions Stock solutions (50 mmol·L^{-1} for Ami and Nor, 0.5 mmol·L^{-1} for Des) were prepared in HPLC-grade methanol and were kept at -4 ℃.

Sample preparation After termination of enzyme reaction, 500 μL of incubation buffer containing human liver microsomal protein, NADPH-generating system and various concentrations of Ami were spiked for 5 mL of IS standard solution and was extracted with diethy ether 6 mL. A 5.5-mL aliquot of the organic layer after shaking vigorously for 3 min and centrifugation for 5 min (1000 × *g*) was evaporated under a gentle stream of N_2 at 37 ℃. Residues were reconstituted in 50 μL or 100 μL of eluent and an aliquot (20 μL) were injected onto the chromatograph.

Chromatography The chromatograph consisted of LC-9A pump, SPD-6AV ultraviolet

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detector, CLS-ODS C18 column (150 mm × 4.6 mm ID, 5 μm particle size), Rheodyne 7161 injector valve, and C-R6A Chromato-Integrator. Except for injector valve (Cotati Ltd, Los Angeles, USA), the other above apparatus were purchased from Shimadzu (Tokyo, Japan). The detector was set at 242 nm. The mobile phase was a mixture of CH_3CN and tetramethyl ethylene diamine 12 mmol · L^{-1} in doubly distilled water (40/60, vol/vol). The pH of the mobile phase was finally adjusted to 5.5 by HAc. The flow-rate was 1.5 mL · min^{-1} and the column temperature was maintained at 25 ℃.

Validation study Standard incubation buffer was prepared by spiking an aliquot of each standard solution (Ami, Nor, Des) into the blank incubation buffer which was incubated with human liver microsomal preparations and NADPH-generating system but without Ami (substrate) as described in sample preparation above. Standard curves were constructed by plotting the amount of Ami or Nor against the peak-height ration of the amount of Ami or Nor to that of the IS with Ami or Nor at 0.02, 0.04, 0.12, 0.2, 0.6, 1.0, 3.0, and 10.0 nmol and IS at 2.5 nmol in the blank incubation buffer. The accuracy and precision of the assay were tested at 0.12, 0.6, and 3.0 nmol of both compounds. The limit of detection was defined at a signal-to-noise ratio of 3:1 by 0.04 AUFS. Concentrations of Ami and Nor in the unknown samples were determined from the peak-height ratio of the calibration samples at 3 different levels within the linear range.

Applications Six human liver specimens with normal histology from patients undergoing partial hepatectomy were used to prepare microsomes by differential centrifugation[9]. Microsomal protein concentration was determined colorimetrically[10]. The incubation buffer contained microsomes 0.2 g · L^{-1}, potassium-phosphate buffer 0.1 mol · L^{-1} (pH 7.4), NADPH-generating system, and various concentrations of Ami with or without Cyp-selective inhibitors in a final volume of 500 μL. The reactions were initiated by adding 200 μL NADPH-generating system (NADP 1.25 mmol · L^{-1}, glucose-6-phosphate 12.5 mmol · L^{-1}, glucose-6-phosphate dehydrogenase 2 kIU · L^{-1}, $MgCl_2$ 12.5 mmol · L^{-1}, and edetic acid 0.25 mmol · L^{-1}). After incubation at 37 ℃ in a shaking water bath for 20 min, the reactions was terminated by adding 200 μL NaOH 4 mol · L^{-1}. The incubation buffers obtained were analyzed at once or stored at −30 ℃ until analysis.

RESULTS

Chromatographic separations The retention times of IS, Nor, and Ami were 5.6, 6.5, and 8.9 min, respectively. No potential interfering peaks were found in the blank incubation buffers. Ami, Nor, and IS gave rapid eluting, fully resolved and sharp symmetrical peaks (Fig 1).

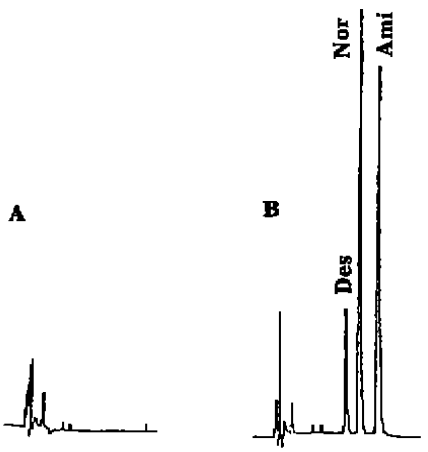

Fig 1. HPLC of (A) extracted blank incubation buffer of human liver microsomes and (B) spiked with Des, Nor, and Ami. Retention time: Des 5.6 min; Nor 6.5 min; Ami 8.9 min.

Validation study The RSD for intra- and inter-day reproducibility ranged from 0.82 % − 4.2 % and 1.7 % − 8.3 % for both Ami and NT, respectively (Tab 1). The relative recovery derived from the found and spiked levels ranged from 94 % − 101 % (Tab 2). Standard curves were made by plotting the peak height

Tab 1. Relative standard deviation (RSD) in determination of amitriptyline (Ami) and nortriptyline (Nor) in human liver microsomes.

Added nmol	Intra-day (n = 5) Ami	Nor	Inter-day (n = 11) Ami	Nor
0.12	1.6	2.0	8.3	5.1
0.6	3.6	1.1	7.5	2.4
3.0	4.2	0.8	7.8	1.7

Tab 2. Relative recoveries of amitriptyline (Ami) and nortriptyline (Nor) in human liver microsomes. $n = 11$, $\bar{x} \pm s$.

	Added/ pmol	Found/ pmol	Relative recovery/ %
Ami	120	115 ± 10	95.8
	600	606 ± 453	101.0
	3 000	2 980 ± 231	99.3
Nor	120	113 ± 6	94.2
	600	580 ± 14	96.7
	3 000	2 970 ± 49	99.1

ration (X) *vs* the spiked concentrations ($\hat{Y}$). Both Ami and Nor were linear over the range of 0.02 – 10 nmol with correlation coefficient (r) of 0.999. The linear regression equation for Ami was $\hat{Y} = 0.3495X - 0.01351$ ($r = 0.9997$) and for Nor $\hat{Y} = 0.3223X - 0.008971$ ($r = 0.9997$). This method showed a limit of detection of 0.01 nmol for both Ami and Nor.

Applications Representative chromatograms from incubation buffer using Ami 0.5 or 10 $\mu mol \cdot L^{-1}$ as substrate are shown in Fig 2. As calculated with standard curve, Ami 0.33 nmol was remained and Nor 37 pmol was produced from Ami 1 $\mu mol \cdot L^{-1}$ after incubation. Eight cytochrome P-450 (Cyp)-selective inhibitors, furafyline, coumarin, sulfphenazole, *S*-mephenytoin, diethyldithiolcarbamate, quinidine, troleandomycin, and ketoconazole were proved to give no chromatographic peaks interfering with those of Ami, Nor, and Des.

DISCUSSION

Unlike plasma and/or urine, the ingredients of incubation buffer for *in vitro* studies of drug metabolism are uncomplicated and definite. Thus, a single-step extraction was employed in this method and good precision was achieved for recovery. It is noted that similar extraction procedures reported previously[2] could make good recovery for plasma and urine sample, and this was further proved by clinical monitoring for antidepressants with this method.

Because Ami, Nor, and Des are all weak basic drugs, their resolution performance was enhanced by using tetramethyl ethylene diamine as a modifier[11] of eluent. In addition, excellent resolution and rapid elution obtained

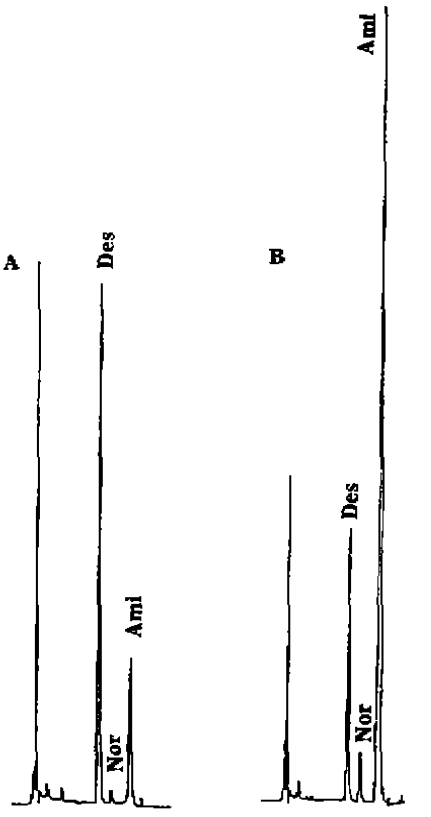

Fig 2. HPLC of extracted incubation buffer of human liver microsomes with Ami 0.5 (A) and 10 $\mu mol \cdot L^{-1}$ (B).

in this method was partially due to this modifier, too[11].

Usually, a great range of substrate concentrations is used in *in vitro* studies[1,7,8]; and due to the limited formation of metabolite *in vitro*, the ratio of metabolite to parent drug in incubation buffer is relatively lower than that in plasma and urine. The ratio of Nor to Ami is about 0.11 in our *in vitro* study but 0.62 in plasma[12]. Therefore, an HPLC method for studying *in vitro* metabolism of drugs must have wide linear range and be more sensitive than that for determining *in vivo* drug concentrations[2-6]. The results of validation study indicate our method meets this demand.

This method is considered to be simple, sensitive, and convenient and thereafter has been successfully utilized in our *in vitro* studies on identification of Cyp isoforms responsible for Ami *N*-demethylation.

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反相高压液相色谱法测定体外人肝微粒体孵育液中阿米替林及其代谢产物去甲替林[1]

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关键词 阿米替林; 去甲替林; 高压液相色谱法; 肝微粒体

目的: 建立同时测定体外微粒体中阿米替林及其 *N*-去甲基代谢产物去甲替林的反相高效液相色谱紫外检测法. **方法**: 含微粒体蛋白和 NADPH 发生系统及药物的孵育液在加入 NaOH 终止反应后, 再加入地昔帕明作内标并以乙醚进行萃取, 然后以反相 ODS 柱分离, 在 242 nm 处以紫外检测器进行检测. **结果**: 孵育体系中没有干扰峰出现, 阿米替林和去甲替林洗脱快, 分离好, 线性范围均为 0.02 – 10.0 nmol, 最低检测限均为 0.01 nmol, 相对回收率为 94 % – 101 %, 变异系数小于 8.3 %. **结论**: 本法快速, 灵敏并且处理过程简单, 可用于体内外阿米替林的代谢研究.