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Short communication

Rapid microsample analysis of imipramine and desipramine by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A rapid and highly sensitive HPLC assay method was developed to measure small amounts of imipramine and its major metabolite, desipramine. The assay involved simple extraction procedures using clomipramine as the internal standard. The mobile phase consisted of acetonitrile (60%) and 0.01 *M* triethylamine in distilled water (40%) with the pH adjusted to 3.0. Separations were achieved on a C_{18} column and the effluent measured for UV absorption at 260 nm. The chromatographic separation was excellent, with no interference from endogenous serum constituents. This assay was suitable for measuring drug concentrations in the range of 10–1000 ng/ml using a 0.1-ml serum sample. The method was applied to a drug disposition study in transgenic mice with increased plasma α_1-acid glycoprotein.

1. Introduction

Imipramine is a tricyclic antidepressant commonly used in the treatment of reactive and endogenous depression, involutional melancholia and senile depression. Desipramine is an aliphatic N-demethylated metabolite of imipramine which is present in plasma as a major metabolite at steady-state. As with the parent drug, desipramine possesses antidepressant activity. Therefore, monitoring plasma levels of both imipramine and desipramine is beneficial in interpreting the relationship between plasma drug concentrations and the therapeutic outcome.

To date, several assay methods have been reported for the measurement of imipramine and its metabolites by gas chromatography [1,2], enzymatic immunoassay [3,4] and HPLC with UV detection [5–10], electrochemical detection [11] or fluorescence detection [12–14]. Although these HPLC methods provide good assay sensitivity for imipramine and its metabolites, the methods involve time-consuming drug extraction procedures and require a relatively large volume of biological samples, typically 1–2 ml of plasma or urine [5–14]. Unlike other HPLC assay methods, the present method requires only 0.1 ml of serum sample volume, involves a single extrac-

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tion procedure and provides excellent yields, high specificity and relatively short retention times. The applicability of the assay method was demonstrated in the study of the pharmacokinetic disposition of imipramine in transgenic mice with increased levels of plasma α_1-acid glycoprotein (AAG).

2. Experimental

2.1. Chemicals and standard solutions

Imipramine, desipramine, clomipramine (all as hydrochloride salts), triethylamine and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol and hexane (all HPLC grade) were obtained from Baxter Healthcare (Muskegon, MI, USA) and phosphoric acid 85% from Malinckrodt (Paris, KY, USA). Standard stock solutions of imipramine (1.0 μg/ml), desipramine (1.0 μg/ml) and clomipramine (5.0 μg/ml) (all as free base) were prepared in methanol and were stored frozen at −20°C until analysis.

2.2. Chromatography

Chromatographic analysis was performed on a Shimadzu component system consisting of a LC-10AS pump, CR501 integrator, SDP-10 AV UV–Vis detector (Shimadzu, Columbia, MD, USA) and an RH 7215 Rheodyne semi-auto manual injector. A Microsorb MV (Rainin, Woburn, MA, USA) octadecyl column (15 × 0.46 cm I.D., 5 μm) was used in the assay. The mobile phase consisted of 60% acetonitrile and 40% 0.01 *M* triethylamine in distilled water, with the pH adjusted to 3.0 by dropwise addition of 85% phosphoric acid. The mobile phase was filtered via a Millipore (Milford, MA, USA) system and degassed. The flow-rate was set at 1.0 ml/min and the effluent was monitored for UV absorption at 260 nm.

2.3. Standard curve and extraction procedures

Standard curves were constructed using control serum harvested from inbred mice (C57BL/6) via cardiac puncture. Aliquots of the stock solutions of imipramine and desipramine (each 1.0 μg/ml in methanol) were added to 75 × 12 mm, disposable, snap-capped borosilicate glass tubes containing 0.1 ml of serum to achieve drug concentrations equivalent to 10, 25, 50, 100, 250, 500 and 1000 ng/ml serum. Following addition of 25 μl of the internal standard (clomipramine 5.0 μg/ml in methanol), the tubes were vortex-mixed for 30 s. To this mixture were added 100 μl of 5.0 *M* sodium hydroxide and 2 ml of hexane. The tube was capped, vortex-mixed for 30 s and centrifuged for 3 min at 3000 *g* (Heraeus Sepatech, Am Kalkberg, Germany). The organic layer was transferred into a fresh tube and dried under nitrogen gas at 20°C using an N-Evap Evaporator (Organomation, Berlin, MA, USA). The resulting residue was reconstituted with 50 μl of mobile phase prior to injection. The samples were vortex-mixed for 30 s and an aliquot (20 μl) was injected onto the chromatographic system. Standard curves were obtained daily by plotting the peak-area ratios of the drug to internal standard against drug concentrations. For sample drug analysis, 0.1 ml of serum was subjected to extraction and drug concentrations were determined from the linear regression line of the standard data.

2.4. Recovery and variability studies

For the determination of the extraction yield, stock solutions were added to 0.1 ml of control serum to yield drug concentrations of 100 and 1000 ng/ml for imipramine and desipramine and 1000 and 5000 ng/ml for clomipramine. The drug was then extracted as described above. The peak areas of the extracted and unextracted samples were compared. Recovery was determined as the mean (±S.D.) of four samples. Intra- and inter-day variabilities were determined at 25, 100 and 1000 ng/ml drug concentrations ($n = 4$).

2.5. Animal experiments

Adult (2–4 months old, male and female) control mice (C57BL/6) and transgenic mice [(C57BL/6 × DBA)F_2 × C57BL/6] with elevated AAG levels were used in the drug disposition

study. A detailed description of the production and characterization of these transgenic mice was reported previously [15]. These transgenic mice were produced utilizing an AAG gene construct derived from a 9.5-kb rat genomic clone containing the entire coding region along with 4.7-kb of 5′ flanking sequence. Briefly, a solution containing the DNA was injected into male pronuclei of $(C57BL/6 \times DBA/2)F_2$ embryos which were then implanted into the uterus of pseudopregnant females and allowed to develop to term. The transgenic mice of this particular line exhibited AAG levels 8.6-fold elevated over those found in inbred controls (0.26 mg/ml) as determined by rocket immunoelectrophoresis.

To study the effect of altered AAG on the pharmacokinetic disposition of imipramine, a 5 mg/kg dose of imipramine (as a free base) dissolved in saline (1.5 mg/ml) was administered by tail vein injection to each group of control and transgenic mice (~20–30 g). Following injection, blood samples (~0.7 ml) were taken by cardiac puncture at 0, 5, 15, 30 min and 1, 2, 4, 6 and 8 h ($n = 3$–4 for each time point). Serum samples were then harvested and stored at −20°C until drug analysis. Drug concentrations were determined as the mean of duplicate samples. The drug elimination half-life was estimated by linear regression of the terminal linear phase of the mean serum drug concentration–time profile in each group of mice.

3. Results and discussion

Typical chromatograms of blank serum, serum spiked with imipramine and desipramine and serum samples obtained after intravenous injection of 5 mg/kg imipramine are shown in Fig. 1. Desipramine, imipramine and clomipramine were eluted with retention times of 5.0, 6.0 and 8.3 min, respectively. Chromatographic separation was excellent, with no interfering peaks from endogenous serum constituents. The drug concentration was linearly related to drug vs. internal standard area ratios over the range studied, with a correlation coefficient > 0.999. Typically, the equations obtained by the least-squares linear regression were $y = 0.0010x +$ 0.0011 for imipramine and $y = 0.0010x + 0.0032$ for desipramine. The signal-to-noise ratio was 10.8 for imipramine and 4.6 for desipramine for the 25 ng/ml sample. The UV_{max} for imipramine occurs at 215–220 nm, with a secondary absorption peak occurring at 250–260 nm, whereas the UV_{max} for desipramine occurs at 250 nm. Previously published HPLC methods monitored the UV absorption of both imipramine and desipramine at 215 nm [9], 220 nm [10] or 254 nm [5–8]. When the effluents were measured at 215 nm or 220 nm in our study, there were interfer-

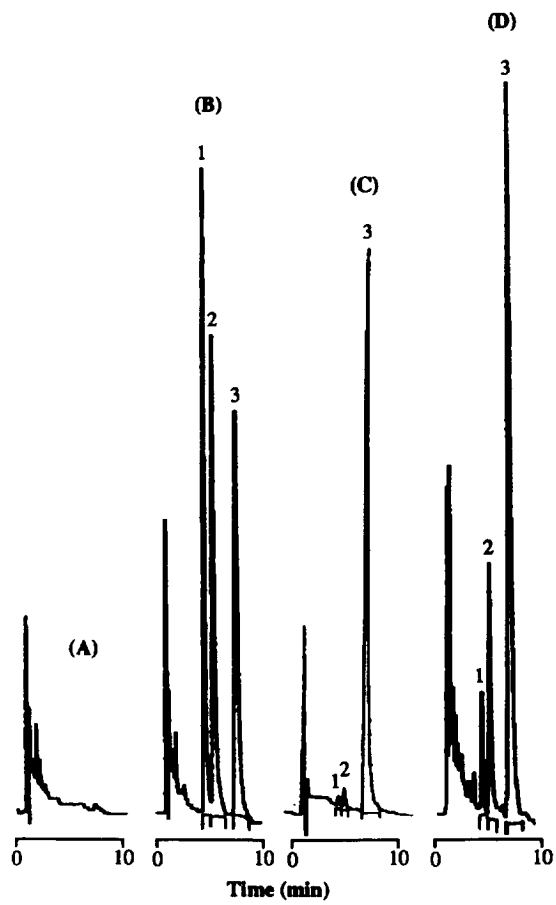

Fig. 1. Representative chromatograms of (A) blank serum, (B) blank serum containing 1 μg/ml of imipramine and desipramine, (C) blank serum containing 25 ng/ml of imipramine and desipramine, and (D) serum sample (imipramine = 435 ng/ml; desipramine = 118 ng/ml) obtained after a 5-mg/kg imipramine i.v. dose. Peaks: 1 = desipramine ($t_R = 5.0$ min); 2 = imipramine ($t_R = 6.0$ min); 3 = clomipramine ($t_R = 8.3$ min, internal standard).

Table 1
Recovery of imipramine, desipramine and clomipramine ($n = 4$)

Drug	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)
Imipramine	1000	94.0 ± 7.6
	100	99.4 ± 2.7
Desipramine	1000	98.7 ± 3.6
	100	94.7 ± 18.0
Clomipramine	5000	98.5 ± 3.8
	1000	89.6 ± 2.0

ing peaks after either single or double extraction with various organic solvents including methylene chloride, toluene and hexane. In order to avoid these interfering peaks, the UV absorption wavelength of 260 nm was selected for subsequent assays. At this wavelength, there was no interfering peak resulting from single extraction using hexane and the assay provided excellent sensitivity. Other previously reported HPLC methods used biological sample volumes of 1–2 ml [5–14]. Since the present assay requires only 0.1 ml of sample volume, this method appears suitable to be used to characterize the pharmacokinetic disposition of imipramine and desipramine in cases where biological sample volumes are limited. A chromatographic run time of less than 9 min is shorter than other HPLC methods with a run time of 15–30 min [5–11]. This assay also provided excellent extraction yields (>89.6%) for all three drugs (Table 1) with little inter- and intra-day variability (Table 2).

The applicability of the assay method was demonstrated in the study of the pharmacokinetic disposition of imipramine in transgenic mice with increased serum AAG levels. AAG is a serum protein primarily responsible for the binding of weakly basic drugs, including imipramine [16]. Serum levels of AAG are elevated in a number of pathophysiological conditions such as depression, myocardial infarction and surgery [17]. Increases in serum AAG have been known to significantly alter the tissue distribution and elimination of drugs via altered serum protein binding [18]. Fig. 2 shows mean concentration–time profiles of imipramine and desipramine after a single intravenous injection of imipramine (5 mg/kg) to transgenic and control mice. The mean elimination half-life of imipra-

Table 2
Intra-day and inter-day variability for imipramine and desipramine

Theoretical concentration (ng/ml)	Concentration found[a] (mean ± S.D., $n = 4$) (ng/ml)	
	Imipramine	Desipramine
Intra-day variability		
1000	992.4 ± 31.1 (3.1)	949.0 ± 39.5 (4.2)
100	102.9 ± 5.8 (5.6)	103.2 ± 7.8 (7.6)
25	26.6 ± 4.4 (16.3)	23.7 ± 3.9 (16.5)
Inter-day variability		
1000	1015.4 ± 30.6 (3.0)	1002.4 ± 45.2 (4.5)
100	92.1 ± 8.4 (9.1)	98.4 ± 10.4 (10.5)
25	28.3 ± 3.7 (13.1)	23.9 ± 1.1 (4.6)

[a] Values in parentheses are coefficients of variation (%).

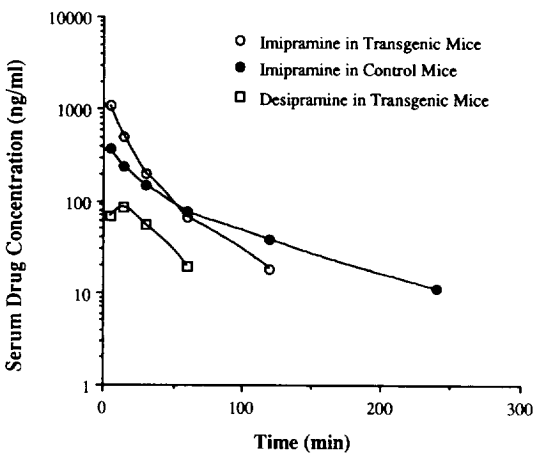

Fig. 2. Mean (±S.D.) serum imipramine concentration–time profiles following i.v. injection (5 mg/kg) to transgenic and control mice.

mine was reduced in transgenic mice (35.0 vs. 65.3 min) compared with control mice. The volume of distribution of imipramine determined as i.v. dose divided by the plasma drug concentration extrapolated to time zero (dose/conc.) was also reduced (5.5 vs. 19.8 l/kg) in transgenic mice due to increased serum drug binding in these animals. As with the parent drug, desipramine concentrations were higher in transgenic mice than in control mice. Desipramine concentrations in nontransgenic mice were not shown because the levels were too low to be quantitated. The metabolite levels may become more clinically meaningful upon multiple dosing since the metabolite may accumulate in the body more extensively than the parent drug due to the longer half-life [19].

In summary, a sensitive and simple reversed-phase HPLC method with UV detection was developed for imipramine and desipramine. This assay method was found to be suitable for drug analysis where sample volume is limited.

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