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DETERMINATION OF 2-HYDROXYDESIPRAMINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple reversed-phase high-performance liquid chromatographic assay of 2-hydroxydesipramine (2-OH-DES) in plasma is described, using 2-hydroxyimipramine (2-OH-IMI) as the internal standard. Extraction of the plasma samples by methylene chloride—isoamyl alcohol was followed by back-extraction of 2-OH-DES into acidic phosphate buffer. Precautions include silanizing test tubes and rinsing pipettes to minimize adsorptive loss, and washing with extraction solution to eliminate chromatographic interference peaks. Analyses were carried out by using a high carbon load C-18 column (15%) with phosphate buffer—acetonitrile as the mobile phase at 43°C. Detection at 254 nm was monitored at extended attenuation of 0.001 or 0.002 a.u.f.s. Peak height ratios of 2-OH-DES/2-OH-IMI were linearly correlated with 2-OH-DES concentration between 10 and 100 ng/ml of plasma. Detection limit was 3 ng. Coefficients of variation for within-run and day-to-day studies were 2.2% and 5.0%, respectively. A significant amount of 2-OH-DES was identified from the plasma extract of a psychiatric patient taking a daily dose of desipramine. This assay may be used for monitoring of 2-OH-DES in evaluating clinical side effects and for pharmacokinetics studies.

INTRODUCTION

Plasma tricyclic antidepressant (TCA) levels have proven to be useful for correlation with clinical response in patients [1]. Tricyclics are metabolized in the liver to both active and inactive metabolites prior to elimination [2]. Hydroxylated metabolites of desipramine and imipramine are examples of such active metabolites. 2-Hydroxydesipramine (2-OH-DES) has been shown to produce cardiotoxicity in dogs [3]. Thus, either clinical responses or side

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effects may depend on levels of parent TCA and such active metabolite as 2-OH-DES.

Hydroxylated metabolites of desipramine and imipramine were quantitated by several methods. Stiller et al. [4] utilized alkaline flame-ionization gas chromatography to monitor plasma levels of imipramine and desipramine and their hydroxylated metabolites. Sutfin and Jusko [5] reported a normal-phase high-performance liquid chromatographic (HPLC) assay of these same drugs and metabolites. A fluorescence detector was used to achieve a sensitivity of 1 ng for each drug in 1 ml of plasma. More recently, Suckow and Cooper [6] used reversed-phase paired-ion liquid chromatography and a highly sensitive electrochemical detector to monitor these drugs with a sensitivity of less than 1 ng/ml of plasma. Godbillon and Gauron [7] analyzed clomipramine, imipramine and their metabolites using a silica gel column with ethanol—hexane—dichloromethane—diethylamine as the eluent and a 254-nm detector. Detection limits ranged from 5 to 10 ng. Fekete et al. [8] used a reversed-phase column with water—ethanol—decylamine at pH 9.5 or pH 11 as the mobile phase for the measurement of chlorpromazine, imipramine and their metabolites. Detection limits ranged from 1 to 3 ng.

The present study is a continuation of efforts within our laboratory to develop methodologies for tricyclics level determination [9, 10]. Since attempts to assay both desipramine and 2-OH-DES using the previously published procedure [9] were not successful as explained in the discussion later on, a reversed-phase HPLC assay for 2-OH-DES has been developed using a high carbon load C-18 column and a 254-nm UV detector.

EXPERIMENTAL

Reagents

Acetonitrile, methanol and methylene chloride were ultraviolet grade, distilled in glass, obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was double distilled in glass. 2-OH-DES and 2-hydroxyimipramine (2-OH-IMI) were gifts from Dr. Albert A. Maniam, National Institute of U.S.A.). Water was double distilled in glass. 2-OH-DES and 2-hydroxyimipramine (2-OH-IMI) were gifts from Dr. Albert A. Maniam, National Institute of Mental Health (NIMH) (Rockville, MD, U.S.A.). Sodium carbonate was reagent grade from Mallinckrodt (St. Louis, MO, U.S.A.). Methylene chloride—isoamyl alcohol (98:2) was washed with 0.05 *M* phosphate buffer, pH 2.5. Sodium carbonate buffer, (1 *M*, pH 11.0) was washed with the above methylene chloride—isoamyl alcohol.

Mobile phase

To 2 l of distilled water, potassium dihydrogen phosphate (13.68 g) was added and the pH adjusted to 4.7 with diluted potassium hydroxide. The solution was filtered and kept at 4°C. Prior to the analysis, the phosphate solution was mixed with acetonitrile (75:25), followed by degassing.

Standards

A primary stock solution of 2-OH-DES (0.1 mg/ml free base) was prepared

by dissolving the powder in distilled water in a silanized 10-ml volumetric flask and stored at 4°C. For the preparation of calibration standards, a working aqueous stock solution of 1 μg/ml was prepared. A stock solution of the internal standard, 2-OH-IMI, was prepared by dissolution in methanol in a silanized volumetric flask. This solution was kept at −20°C. For spiking purposes, a working standard solution of 1 μg/ml was prepared.

High-performance liquid chromatography

The liquid chromatograph consisted of a Model 6000A pump, a Waters guard column, 2.3 cm × 3.9 mm, packed with Bondapak/Porasil particles, a U6K injector and a Model 440 254-nm detector (Waters Assoc., Milford, MA, U.S.A.). The column was a Partisil ODS-2 reversed-phase column, 250 mm × 4.6 mm (Whatman, Clifton, NJ, U.S.A.). The column packing consisted of 10-μm silica gel with 15% of the surface hydroxyls bonded with octadecyl groups. Detector attenuation was set at either 0.01 or 0.02 a.u.f.s. with the recorder input voltage set at 1 mV. In effect, the attenuation was extended to 0.001 or 0.002 a.u.f.s. The analysis was carried out by using the previously prepared mobile phase at a flow-rate of 2.7 ml/min and at an elevated temperature of 43°C.

Sample extraction

To a series of silanized test tubes, each containing 2 ml of plasma, 0, 10, 20, 40, 80, and 100 ng of 2-OH-DES were added per ml of plasma. In addition, a psychiatric patient plasma and five previously spiked plasma samples (20 ng/ml) were also included for checking the precision of this assay. To these samples, 75 ng/ml of 2-OH-IMI were added, followed by 2 ml of carbonate buffer (1 *M*, pH 11) and 10 ml of methylene chloride—isoamyl alcohol (98:2). The sample tubes were capped, shaken for 15 min and spun for 10 min. The upper, aqueous phase was discarded, and the lower organic phase transferred into another silanized test tube, containing 400 μl of phosphate buffer (0.05 *M*, pH 2.5, previously washed with hexane). The extraction was completed by shaking for 15 min and centrifuging for 5 min. The lower phase was transferred by rinsed pipettes [methylene chloride—isoamyl alcohol (98:2)] to another set of silanized test tubes, and 350 μl were injected for HPLC analysis.

Quantitation

Peak height ratios of 2-OH-DES to 2-OH-IMI were plotted against concentrations of 2-OH-DES. The concentrations of 2-OH-DES of precision studies and patient's sample were estimated from these plots.

Recovery

Percentage recoveries of 2-OH-DES were established for the concentrations of 20 and 50 ng/ml. At both concentrations, five determinations were made. These samples were spiked with internal standard, followed by extraction as described previously. Then, the peak height of extracted 2-OH-DES was compared to that of a known amount for recovery percentage estimation.

Interferences

Interferences were checked by comparing the capacity factor, k', of some commonly used drugs with those of 2-OH-DES and 2-OH-IMI.

RESULTS

Measurements of 2-OH-DES concentration in plasma were achieved by using a rapid three-step extraction, followed by a reversed-phase HPLC analysis. Fig. 1A shows the chromatogram of a blank (unspiked) plasma sample spiked with the internal standard, 2-OH-IMI. Fig. 1B shows the chromatogram of a plasma sample spiked with 20 ng of 2-OH-DES per ml of plasma, and the internal standard. The two peaks were well resolved, and the capacity factors of 2-OH-DES and 2-OH-IMI were 6.20 and 8.90, respectively. The sensitivity for a signal-to-noise ratio of 5:1 was 3 ng. Blank plasma sample (Fig. 1A) did not show any endogenous interference peaks with 2-OH-DES. Each analysis was completed within 13 min.

Calibration studies showed that the peak height ratios of 2-OH-DES/2-OH-

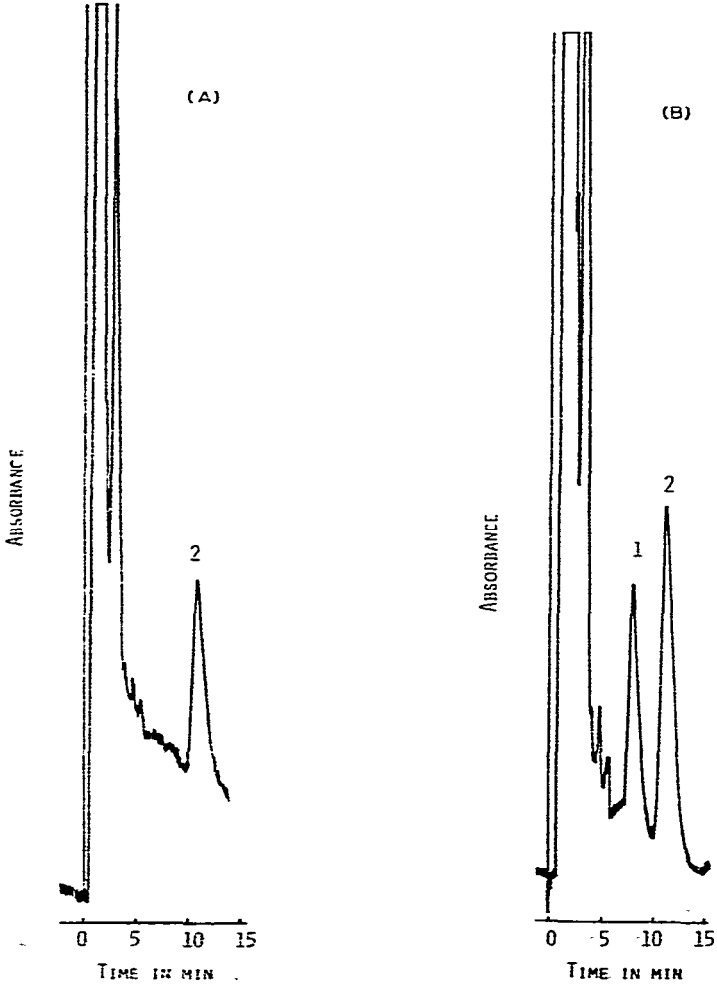

Fig. 1. Chromatograms of (A) extract of drug-free plasma spiked with internal standard, 2-OH-IMI, and (B) extract of plasma spiked with 20 ng of 2-OH-DES per ml of plasma. Peaks: 1 = 2-OH-DES, 2 = 2-OH-IMI.

TABLE I

CAPACITY FACTOR, k', OF COMMON DRUGS CHECKED FOR INTERFERENCE

Drug	k'	Drug	k'
Acetaminophen	0.00	Doxepin	a
Codeine	0.65	Cimetidine	a
Phenobarbital	2.16	Desipramine	a
2-Hydroxydesipramine	6.20	Nortriptyline	a
Phenytoin	6.85	Propoxypene	a
Meperidine	7.03	Imipramine	a
Pentobarbital	7.05	Amitriptyline	a
2-Hydroxyimipramine	8.90	Diazepam	a
Secobarbital	10.20	Chlorpromazine	a
Oxazepam	12.90	Clomipramine	a
Lorazepam	a*	Perphenazine	a
Flurazepam	a	Thioridazine	a
Chlordiazepoxide	a	Trifluoperazine	a
		Prochlorpherazine	a

*a, Capacity factor greater than 12.90.

IMI were linearly correlated with concentrations between 10—100 ng of 2-OH-DES per ml of plasma. The correlation coefficient was 0.9994 with a slope of 0.0321 and an intercept of 0.0868. Within-run precision was estimated by the determination of five 2-ml plasma samples containing 20 ng of 2-OH-DES per ml of plasma. Coefficient of variation was 2.2%. This experiment was repeated five times over a period of a month in order to evaluate the day-to-day variability, and the coefficient of variation was 5.0%.

Recovery of 2-OH-DES was estimated by using five 20 ng/ml and five 50 ng/ml samples. Peak height of the 2-OH-DES standard, either 20 or 50 ng, was compared to that of the extracted 2-OH-DES. The percentage recoveries were 66% and 68% for the 20 ng/ml and 50 ng/ml samples, respectively.

Table I lists the capacity factors, k', of about 20 common drugs as analyzed by our chromatographic system. None of these drugs interfere with our assay of 2-OH-DES or 2-OH-IMI. As a result of the high carbon load of this Partisil-10 ODS-2 column, the other TCA, such as desipramine and imipramine were well retained by the column with a k' greater than 10.

To evaluate the applicability of this assay to clinical and pharmacokinetics studies, a plasma sample from a psychiatric patient taking a daily dose of 150 mg of desipramine was measured by the procedure. Fig. 2 shows the chromatogram of plasma extract from this patient. Concentration of 2-OH-DES was estimated to be 51 ng/ml. The desipramine concentration, determined by our previously published procedure [9], was 80 ng/ml.

DISCUSSION

Preliminary studies of the simultaneous assay of 2-OH-DES and desipramine using the published procedure [9] were not successful as explained later on in this section. The approach of development of a new 2-OH-DES assay was chosen, resulting in a systematic search for optimization of extraction proce-

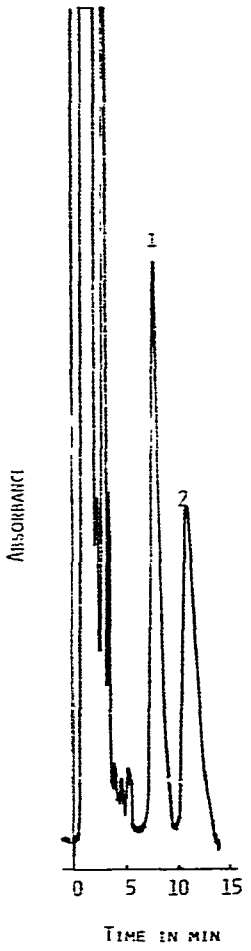

Fig. 2. Chromatogram of a patient plasma extract, showing 2-OH-DES at a concentration of 51 ng/ml. Plasma sample was taken 8 h post-ingestion.

dures and chromatographic conditions. Based upon our previous experience, the following precautions were taken in order to avoid adsorptive losses and interference peaks from extraction reagents. To minimize adsorptive losses, the test tubes, volumetric flasks and transfer pipettes were either silanized or rinsed with the extraction solution [methylene chloride—isoamyl alcohol (98:2)]. To avoid interference peaks from the extraction reagents, the washing of sodium carbonate buffer and methylene chloride—isoamyl alcohol solutions was carried out according to the procedures of the Experimental section. That these steps ensured precisions in replicate determinations is evident from the small coefficient of variation obtained.

Another important aspect of the assay development was the chromatography. Due to the obvious advantages of reversed-phased HPLC for biological assays, the initial effort was concentrated on using a μBondapak C_{18} column with a 254-nm detector as described in the published assay [9]. However, attempts at retaining the polar 2-OH-DES with k' between 2 and 10 and achiev-

ing resolution from endogenous interference were not successful. Thus, it became necessary to use a reversed-phase column with a high carbon load in order to obtain the desired retention characteristics and resolution. The column of choice was a Partisil-10 ODS-2 with a 15% carbon load. Because of the higher percentage of C-18, the 2-OH-DES and 2-OH-IMI molecules experienced more interaction with the column packing than that with a 10% carbon load μBondapak C_{18} column. Due to increased interaction, peak broadening occurred. This was overcome by carrying out the separation at an elevated column temperature of 43°C. In contrast to several previous attempts by other workers, the detection mode utilized a readily available 254-nm UV detector. An extended attenuation of either 0.001 or 0.002 a.u.f.s. was used, and the response was linear for 2-OH-DES concentrations of 10—100 ng/ml of plasma. The coefficients of variation for within-run and day-to-day studies were equal or less than 5%. The present assay identified the presence of a significant amount of 2-OH-DES in the plasma of a psychiatric patient.

Since the present procedure involved extraction of 2-OH-DES and 2-OH-IMI from plasma samples, the possibility of simultaneous assay of these two metabolites by modifying the present method was investigated. The approach was the external standard method. Modifications included: firstly, the preparation of plasma calibration standards for both 2-OH-DES and 2-OH-IMI; secondly, quantitative transfer of methylene chloride—isoamyl alcohol; and finally, quantitative injection of the phosphate buffer extract. Preliminary studies show that the calibration curves for both 2-OH-DES and 2-OH-IMI concentrations versus the respective peak heights were linear. This modified method may be used for assaying both concentrations of plasma samples from patients taking imipramine.

Thus, the present study shows that measurement of 2-OH-DES in plasma can be achieved by using a rapid three-step extraction process, and a reversed-phase column. By following precautions to avoid adsorptive loss and extractant interference, this procedure may be used for both pharmacokinetics studies and patient monitoring of 2-OH-DES levels, which may be correlated with clinical side effects.

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