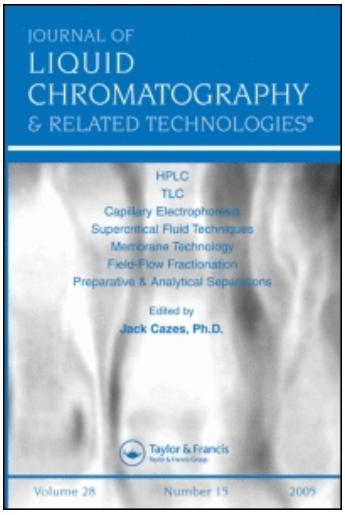

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Reversed Phase High - Performance Liquid Chromatographic Analysis of Tricyclic Antidepressants in Plasma

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To cite this Article Wong, Steven H. Y. and McCauley, Thomas(1981) 'Reversed Phase High - Performance Liquid Chromatographic Analysis of Tricyclic Antidepressants in Plasma', Journal of Liquid Chromatography & Related Technologies, 4: 5, 849 — 862
To link to this Article: DOI: 10.1080/01483918108059979
URL: http://dx.doi.org/10.1080/01483918108059979

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(5), 849-862 (1981)

REVERSED PHASE HIGH - PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF TRICYCLIC ANTIDEPRESSANTS IN PLASMA

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ABSTRACT

A reversed phase HPLC procedure is described for measuring the plasma concentration of four commonly used tricyclic antidepressants (TCA): amitriptyline, desipramine, imipramine and nortriptyline in the range of 25 to 800ng per ml. The procedure involves rapid extraction, and HPLC analysis using a μBondapak C-18 column at 50°C, and a 254nm detector. Coefficients of variation are less than 5% for within run, and 7% for day-to-day experiments. Detection limits are: desipramine - 0.5ng, nortriptyline or imipramine - 0.6ng, and amitriptyline - 0.7ng. Propoxyphene interferes with amitriptyline while chlorpromazine interferes with clomipramine. The procedure is easily adapted for clinical drug monitoring of TCA.

INTRODUCTION

Tricyclic Antidepressant (TCA) measurement has been achieved by a wide variety of methods as recently reviewed by Scoggins, et al.(1) Among the commonly used assays, high - performance liquid chromatography (HPLC) procedures have offered adequate sensitivity, specificity, accuracy and precision in comparison to

other techniques. HPLC procedures generally involve TCA extraction from plasma or serum(2,3), followed by different modes of chromatographic separation and detection. Normal phase separation accounts for the majority of the published procedures (4,5,6). Separations have also been carried out by using reversed - phase column with alkaline mobile phase(9), paired-ion chromatography(10,11), a CN - bonded column(12) and an alkyl phenyl column(13). In some of these procedures, either a variable wavelength UV detector(4) or a fluorescence detector(5) was used to achieve the desired sensitivity of 2 to 10ng of TCA per ml of plasma. For routine quantitation of TCA, there exists a need for a procedure using readily available, highly efficient octadecylsilane column, and a "universal" 254nm UV detector.

The present study was undertaken to develop a procedure for the routine clinical determination of imipramine and amitriptyline, as well as their metabolites, desipramine and nortriptyline in plasma by utilizing rapid TCA extraction procedure. The subsequent analysis was carried out by using a μBondapak C-18 column without ion-pairing, and a fixed 254nm detector.

EXPERIMENTAL

Reagents:

Acetonitrile, methanol and hexane, distilled in glass, ultraviolet grade, were obtained from Burdick and Jackson Laboratories (Muskegon, Mich. 49442).

Water was double distilled in glass. Isoamyl alcohol, Ortho-phosphoric acid and potassium dihydrogen phosphate were "Baker-Analyzed" Reagent grade (Phillipsburg, N. J. 08865).

Imipramine was kindly supplied by Ciba-Geigy Corp. (Summit, N. J. 07901).

Desipramine was kindly supplied by USV Pharmaceutical Corp., (Tuckahoe, N. Y. 10707).

Nortriptyline was kindly supplied by Eli Lilly and Co. (Indianapolis, Ind. 46206).

Amitriptyline was kindly supplied by Merck Sharp & Dohme Research Lab. (Rahway, N. J.).

Mobile Phase:

Potassium dihydrogen phosphate (13.68gm) was dissolved in 2L of water, and adjusted to pH 4.7 with dilute potassium hydroxide. The solution was filtered and stored at $4^{o}C$. Prior to the analysis, the phosphate solution was mixed with acetonitrile to a ratio of 6:4.

Standards:

The stock solutions of the four TCA (1mg/ml free base) were prepared by dissolving their hydrochloride salts in distilled water, and stored at $4^{o}C$. These stock solutions were diluted to 10ng/ml in a silanized vessel for the preparation of calibration standards. Stock solutions, using 0.05% ortho phosphoric acid, were also prepared for recovery studies. The internal standard, clomipramine (1mg/ml) was dissolved in methanol, and stored at

-20°C. A working internal standard solution of 10ng/ml of methanol was prepared for spiking purpose.

Instrumentation:

The chromatograph consisted of a Model M6000A solvent delivery system, Model U6K injector, μBondapak/Porasil guard column, a μBondapak C-18 column, and a Model 440 UV absorbance monitor (254nm). (Waters Assoc., Milford, Mass. 01751) Chromatograms were recorded on a Omniscribe Recorder, set at either 10mV or 1mV full scale. The column temperature of 50°C was maintained by a thermoregulated water bath.

Sample Extraction:

To two milliliters of blank plasma in silanized tubes, 0, 25, 50, 100, 200, 400, and 800ng/ml of each of TCA were added. Five previously spiked plasma samples, at a concentration of about 170ng/ml for desipramine and imipramine, and about 150ng/ml for nortriptyline and amitriptyline were also included for precision studies. To these plasma samples, methanolic clomipramine (800ng/ml) was added, followed by 2ml of 1N NaOH and 5 ml of hexane-isoamyl alcohol (99:1). These tubes were capped and shaken mechanically for 15 minutes, and spun for 5 minutes at (1686 x g). The upper, organic phase was transferred with a rinsed pipette (hexane-isoamyl alcohol, 99:1) to another silanized test tube containing 200μl of 0.05% ortho-phosphoric acid. This acid back-extraction process was completed by shaking

for 15 minutes and centrifuging for 5 minutes. The lower phase was transferred to another silanized test tube, and 50µl was taken for HPLC analysis.

Chromatography:

The chromatographic conditions were: flow rate of 2ml/minute and column temperature of 50°C. The chromatogram was recorded at 10mV input (0.01 or 0.02 AUFS). For "extended" sensitivity study the attenuation was set at 0.01 AUFS, with the recorder input at 1mV, corresponding to "0.001 AUFS" full scale.

Quantitation:

The peak height ratios of each TCA to the internal standard were plotted against their concentrations. The concentrations of the precision study samples and of the patient samples were determined from this plot.

RESULTS AND DISCUSSION

The four commonly used TCA: desipramine, imipramine, nortriptyline, and amitriptyline were extracted from plasma, and well quantitated by the present procedure. Analysis time for each extract was ten minutes. Figures 1A and 1B show the chromatograms of the "blank" and spiked plasma samples containing 25ng of each TCA per ml of plasma. At this concentration, the recovery was estimated to be 65% by comparison with peak height of each of the four TCA/phosphoric acid standards. Figure 1C shows the four TCA peaks (12.5ng each) from the same plasma

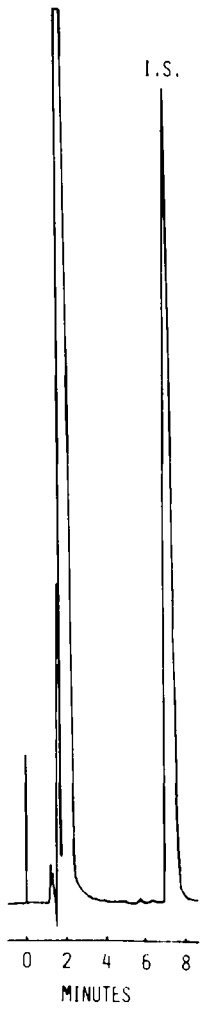

Figure 1A: Chromatogram of a drug free plasma extract

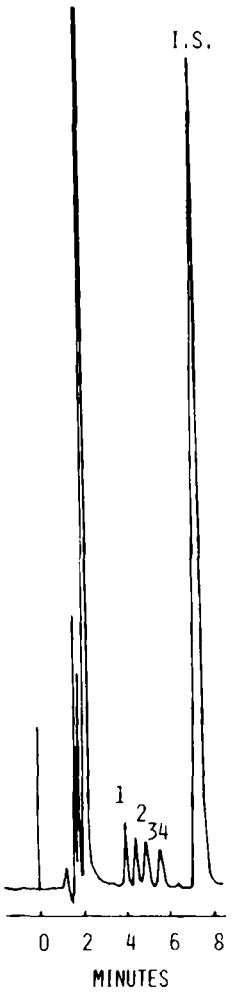

Figure 1B: Chromatogram of a plasma extract containing 25ng of each TCA per ml of plasma. 1. desipramine, 2. nortriptyline, 3. imipramine, and 4. amitriptyline Injection volume = 40µl Attenuation 0.01 AUFS

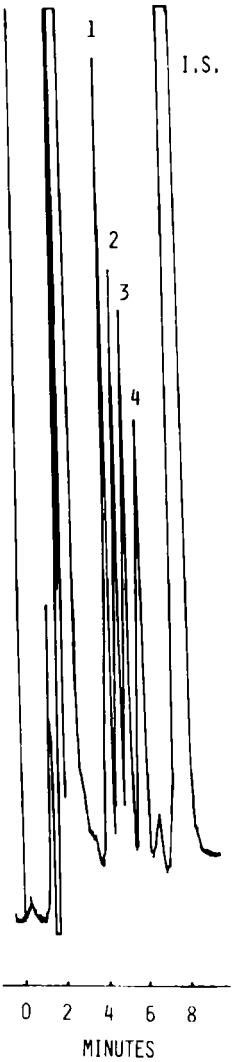

Figure 1C: Chromatogram of the same plasma extract as in Figure B. Injection volume 50µl. Attenuation "0.001" AUFS.

extract by using the extended "0.001 AUFS" attenuation. The detection limits are: (for a signal/noise ratio of 5 to 1) desipramine - 0.5ng, nortriptyline and imipramine - 0.6ng, and amitriptyline - 0.7ng.

Peak height ratios were linearily related to concentration in the range of 25 to 800ng per ml of plasma as shown by Figure 2. Table I shows the linear regression analysis of these curves. Within run reproducibility was established by analysis of five, replicate, "Quality Control" TCA spiked plasma samples. Table II

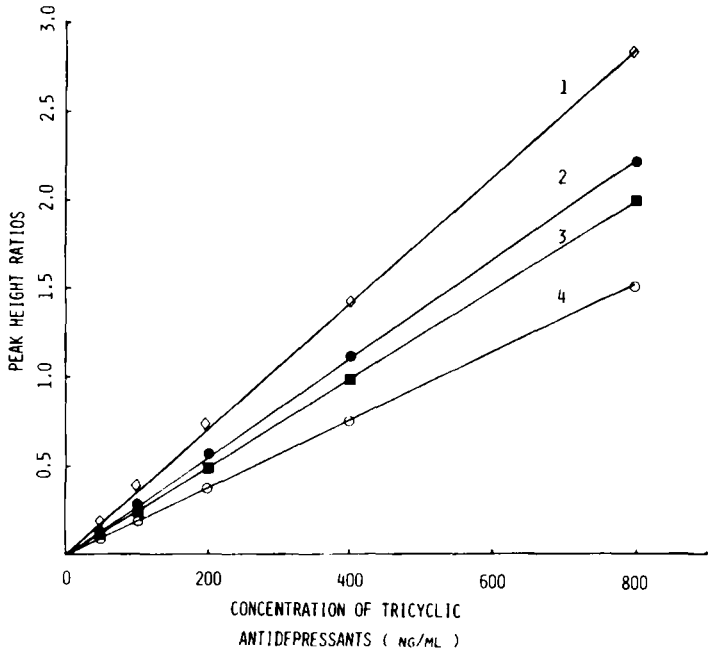

Figure 2: Calibration curves for the four commonly used tricyclic antidepressants. (1) desipramine, (2) nortriptyline, (3) imipramine, (4) amitriptyline.

TABLE I

Linear Regression Data for Calibration Curves

Drug	Slope	y-intercept	Correlation Coefficients
Desipramine	0.0018	0.0002	0.9996
Nortriptyline	0.0015	0.0031	0.9992
Imipramine	0.0013	0.0082	0.9995
Amitriptyline	0.0010	0.0017	0.9997

shows the day-to-day precision, over a 6 week period, estimated from these same samples. Percentage of recoveries for the four TCA were approximately 65% and 75% for 25 and 400ng per ml of plasma respectively.

In developing this procedure, the following guidelines were established. During the sample collection and extraction processes, precautions were taken to minimize the absorption losses, based upon the experience of our laboratory as reported

TABLE II

Within-run and Day-to-Day Precision

	Mean Concn. (ng/ml)	CV_a % Within-run[a]	Day-to-Day[b]
Desipramine	179.8	3.7	6.7
Nortriptyline	156.7	1.0	3.9
Imipramine	176.7	2.7	4.6
Amitriptyline	151.9	1.1	2.9

a. Five measurements. b. Six different days.

by Antal et al (14).These precautions included silanization of extraction test tubes, and volumetric flash (for calibration standards) and rinsing of the transfer pipettes with hexane/ isoamyl alcohol. These steps were essential to maintain reproducibility, especially important for low concentration samples. From our experience, the final TCA/phosphoric acid extracts were stable up to 24 hours, as indicated by the same value of peak height ratios of the same extract. This added flexibility enhances the scheduling in a clinical laboratory.

The optimal chromatographic conditions were found to be: a μBondapak C-18 column with 0.05M potassium monobasic phosphate, at pH = 4.7 / acetonitrile (6:4) with a flow rate of 2ml/minute at 50°C. These were chosen after checking the following parameters: range of pH of the mobile phase: 3.5 to 5.5, range of molarity: 0.001 to 0.05M, phosphate percentage: 80 to 60, and column temperature: 25 to 60°C. With the established chromatographic conditions, tailing was minimal, and overall recovery was comparable to the other methods. Since the mode of separation was reversed-phase, equilibration was reached within 20 to 30 minutes. Interferences were checked by determination of the capacity factor, K' of twenty commonly used drugs as shown in Table III. Propoxyphene interferred with amitriptyline, while chlorpromazine interferred with the internal standard, clomipramine.

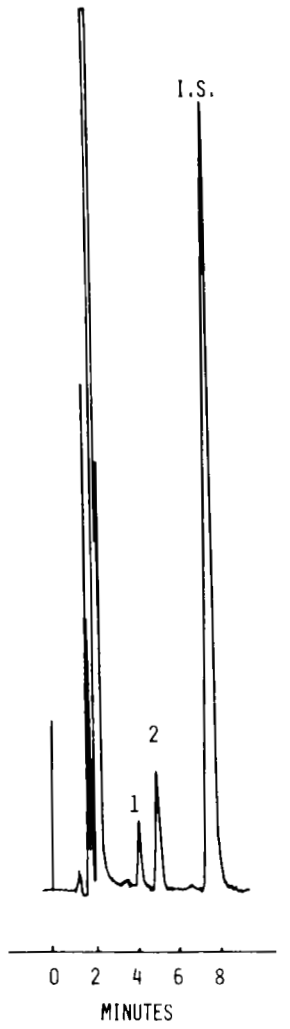

Figure 3: Chromatograms of plasma extracts from a psychiatric patient. (1) desipramine - 28ng/ml (2) imipramine - 61ng/ml.

TABLE III

K' Values of Some Common Drugs Tested for Interference

	K'		K'
Acetaminophen	0.00	Desipramine	2.44
Codeine	0.00	Nortriptyline	2.74
Meperidine	0.12	Imipramine	3.08
Phenobarbital	0.84	Propoxyphene	3.48
Phenytoin	1.40	Amitriptyline	3.49
Pentobarbital	1.56	Diazepam	3.99
Oxazepam	1.79	Chlorpromazine	4.15
Lorazepam	1.88	Clomipramine	4.16
Secobarbital	1.88	Perphenazine	4.92
Flurazepam	2.04	Prochlorperazine	6.04
Chlordiazepoxide	2.18	Thioridazine	8.44
Doxepin	2.20	Trifluoperazine	8.44
Cimetidine	2.36		

The procedure was used to estimate the TCA levels of an alcoholic, depressed patient. Figure 3 shows desipramine (28mg) and imipramine (61mg).

During these past six months, this procedure was successfully carried out in our clinical laboratory to monitor patient's TCA level. The simplicity and precision of this procedure warrant its use in both therapeutic drug monitoring and applications in pharmacokinetics.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of Dr. Paul A. Kramer, and the technical assistance of Mrs. Annarita Erwin for typing the manuscript. Dr. Wong's present address is Department

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