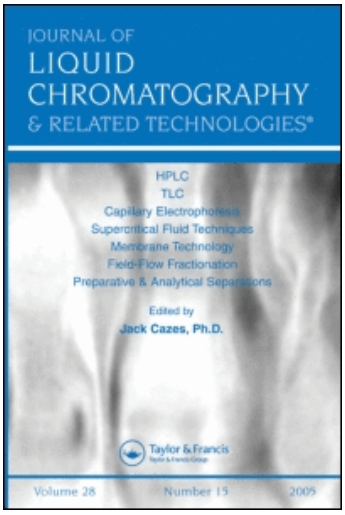

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DETERMINATION OF A TRAZADONE METABOLITE, 1-m-CHLOROPHENYL-PIPERAZINE IN PLASMA BY LIQUID CHROMATOGRAPHY*

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ABSTRACT

A liquid chromatographic assay was developed for monitoring a trazodone metabolite, 1-m-chlorophenylpiperazine (mCPP) in plasma. Alkalinzed plasma was extracted with methylene chloride/isoamyl alcohol (98:2), and back-extracted with diluted phosphoric acid. The extracts were analyzed by a high carbon load, 20%, C-18, 5 um column with a ternary mobile phase at 45°C and detection at 254 nm. Retention volumes of mCPP, the internal standard and trazodone were 22.7, 38.0 and 58.7 ml, respectively. A peak, X, eluded closely with mCPP. Peak height ratios of mCPP/internal standard were linearly correlated to mCPP plasma concentrations between 10 to 100 ug/L. Detection limit was 5 ng and recovery was about 50%. Precision studies showed the within-run and day-to-day coefficients of variation were 4.0 and 5.7%, respectively. Patient samples (n = 6) showed substantial amounts of trazodone but only trace amounts of mCPP.

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INTRODUCTION

Trazodone(TRA), 2-(3-(4-(m-chlorophenyl)-1-piperazinyl)-propyl)-s-triazolo-(4,3a)pyridin-3-(2H)-one hydrochloride, an atypical antidepressant, has been recently approved for clinical application (1-7). Its chemical structure, as shown by Figure 1, is different from the first generation tricyclic antidepressants, such as imipramine and amitriptyline. Its advantage would supposedly include fast on-set of action without any anticholinergic side effect (8). Recent reports showed that side effects of TRA would include cardiotoxicity (9-13), delirium in bulimic patients (14), ejaculatory inhibition (15), priapism (16-18), and mania (19).

Similar to the European experience, TRA is well tolerated in overdose cases (1,6,11,20,21). Patients with plasma levels of TRA ranging from 5 to 25.7 mg/L eventually recovered with supportive therapies. However, one death did occur, possibly due to multiple drugs ingestion (20).

According to previously published studies (2,22-26), TRA is metabolized to 1-m-chlorophenyl- piperazine (mCPP), as shown by Figure 1, and other metabolites. In an animal model study, mCPP accumulated to a higher concentration than that of TRA in rat brain (27). In mice, mCPP reversed an inhibitory action of trazodone on conditioned avoidance responses (28). Other animal studies showed that mCPP is an agonist at central serotonin receptors but is antagonistic of peripheral serotonin receptors (29-31). Since the role of mCPP and its side effects have not been well established for humans, measurement of patient plasma TRA and mCPP concentrations would be useful to understand further their clinical pharmacology.

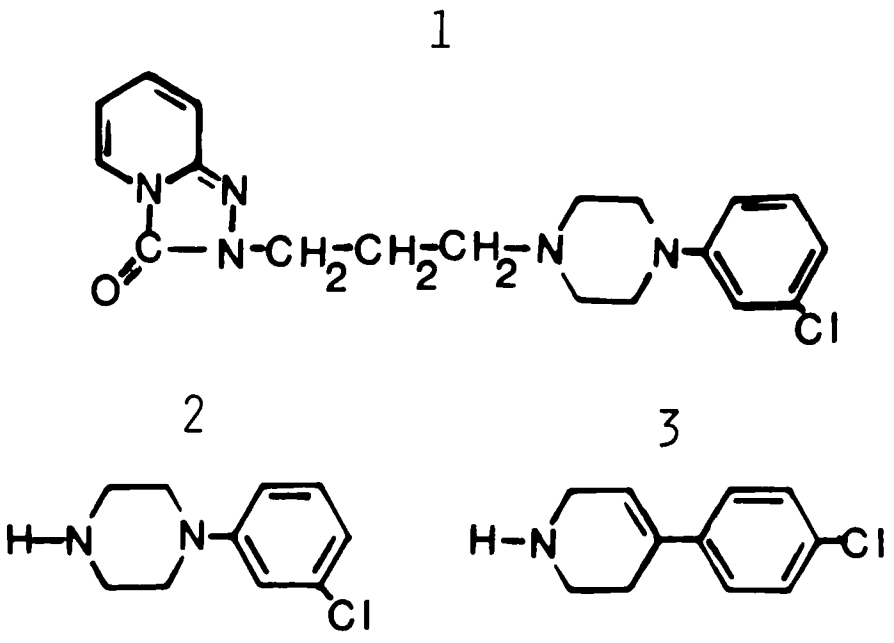

FIGURE 1: Chemical structures of TRA, (1), mCPP (2) and IS (3).

TRA and mCPP plasma concentration had been quantitated by both gas and liquid chromatographic procedures. Caccia, et al. (25) used a gas chromatographic procedure to quantitate both TRA and mCPP plasma concentrations. Abernathy, Greenblatt and Shader recently described a gas chromatographic procedure using a nitrogen phosphorus detector (32). Anderson and Archuleta developed a capillary gas chromatographic procedure using a methyl silicone column (33). Suckow (2) utilized LC - electrochemical detection to quantitate simultaneously TRA and mCPP. Analyses of 5 patients' plasma samples showed substantial amounts of TRA (496-1212 ug/L) but only trace amounts of mCPP (8-28 ug/L). It is important to note that a peak, X, (possibly an unidentified metabolite) eluded

before, and closely with the mCPP peak at about 6 to 7 minutes. Wong, et al. (1) described a procedure for quantitation of TRA with a C-18 column and UV detection at 214 nm. From monitoring 26 patients, the range and mean of TRA were: 73 to 1678 ug/L and 639 ug/L, respectively.

As a continuation of our laboratory's interest in antidepressant clinical pharmacology and monitoring, a simple reversed-phase liquid chromatographic assay was developed to quantitate mCPP in plasma, based upon a three-step sample extraction, followed by analysis with a high carbon load C-18 column with ternary mobile phase and detection at 254 nm. This procedure may be modified to quantitate simultaneously TRA and mCPP concentrations in plasma.

MATERIALS

Reagents

Acetonitrile(ACN), methanol, methylene chloride and tetrahydrofuran(THF) were ultraviolet grade, distilled in glass, obtained from Burdick and Jackson Labs (Muskegon, MI 49442). Isoamyl alcohol, orthophosphoric acid, "HPLC" water, sodium hydroxide, and potassium dihydrogen phosphate were "Baker-Analyzed" reagent grade, obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ 08856). Trazodone metabolite, 1-m-chlorophenylpiperazine dihydrochloride (mCPP), and the internal standard 4-(p-chlorophenyl)-1,2,3,6- tetrahydropyridine monohydrochloride (IS) were obtained from Aldrich (Milwaukee, WI 53233).

Standards

Primary stock solutions of mCPP and IS (1 gm/L) were prepared individually as follows: 13.6 mg of mCPP or 11.8 mg of IS was dissolved separately in 10 mL of MeOH inside a silanized volumetric flask. Working mCPP plasma stock solution (10 ng/uL) was prepared by transferring 100 uL of the above primary stock solution to a 10 mL silanized volumetric flask. Methanol was then evaporated by passing nitrogen. Residual mCPP was redissolved in 10 mL of "drug-free" plasma: analyses of plasma extract, performed as described later, did not show any chromatographic peak overlapping with mCPP. Aliquots were transferred and kept frozen in conical polypropylene vials. Working IS stock solution (1 ng/uL) was prepared by mixing 10 uL of the above primary IS stock with 10 mL of methanol.

Quality control samples containing mCPP (50 ug/L) were prepared as follows: 100 uL of the primary stock solution were mixed with 10 mL of methanol, and 500 uL of this stock solution was added to a 100 mL silanized volumetric flask. Methanol was evaporated by passing nitrogen. Then, 100 mL of "drug-free" plasma was added to the mark. After thorough mixing, 2 mL aliquots were transferred to a silanized test tube, and kept frozen for precision studies and for later analysis.

Mobile Phase

To 2 L of "HPLC" water, 54.72 g of potassium dihydrogen phosphate was added, and pH was adjusted to 4.7 with diluted potassium hydroxide. After filtration, the solution was kept at

4°C. Prior to analysis, the phosphate solution, THF and ACN were degassed separately and mixed according to the following ratio (90:5:5).

Instrumentation

The liquid chromatograph consisted of a Constantmetric III Pump (LDC, Rivera Beach, FL 33404), a Model 7125 injector with a 500 uL loop (Rheodyne, Berkeley, CA 94710), and a Model 440-254 nm detector (Waters Assoc., Milford, MA 01757). The columns included a MC-18 (4.6 mm x 25 cm) packed with 20% carbon load, 5 um particles (E.S. Industries, Marlton, NJ 08053) and a uBondapak C-18 (4.6 mm X 30 cm) packed with 10% carbon load, 10-um particles (Waters Assoc.). Each of the above columns was coupled to a guard column (3.9 mm x 2.3 cm) packed with Bondapak/Corasil C-18 (Waters Assoc.). The column temperature was maintained at 45°C by a water bath. And chromatograms were recorded with an Omniscribe recorder (Houston Instruments, Austin, TX 78753).

Sample Collection

In order to assess steady state "trough" TRA and the corresponding mCPP plasma concentrations, blood samples were obtained from six depressed patients, treated with trazodone (50 - 300 mg bid or tid) for at least three days, either half an hour before the next dose or 12 hours post-ingestion. Blood was collected by using lavender top Vacutainer tubes (Becton-Dickinson, Rutherford, NJ 07070), containing EDTA but without a plasticizer, tris (2-butoxylethyl)phosphate in the rubber stopper. The clinical efficacy of this device for monitoring tricyclic antidepressants and propranolol was established from two previous studies (34,35).

Sample Extraction

For preparing calibration standards, 0,20,50,100,200 ng of mCPP was added to two milliliters of "drug-free" plasma in silanized test tubes. To these "standards", "quality controls" and 2 mL aliquots of patient plasma, 80 ng of IS working stock solution was added, followed by 2 mL of 2N NaOH and 5 mL of methylene chloride/isoamyl alcohol (98:2). For extraction, these tubes were rotated for 10 minutes and centrifuged for 10 minutes. The upper aqueous layer was aspirated. With a silanized pipet, the emulsion in the lower organic layer was stirred and deposited to the side wall to yield a clear solution. With another silanized pipet, the cleared organic layer was transferred to another silanized tube, taking care not to transfer any emulsified material in the process. The organic phase was then extracted with 200 uL of 0.05% phosphoric acid (0.05M, pH = 2.5) by rotation and centrifugation. The upper dil. phosphoric acid bubble, containing the extracted mCPP, TRA and IS, was transferred to another silanized tube. Nitrogen was passed gently for 10 minutes to evaporate any "carry-over" organic. Then the total extract (about 150-170 uL) was injected into the chromatograph.

Chromatographic Parameters

The parameters were: flow rate = 1.5 mL/min.; column temperature = 45^{o}C; detection wavelength = 254 nm; attenuation = 0.001 AUFS (achieved by setting the attenuation at 0.01 AUFS with recorder input at 1 mV instead of the "normal" 10 mV).

Quantitation and Statistical Analysis

Peak height ratios of mCPP to IS from the "standards" chromatograms were plotted against mCPP concentrations by using

linear regression of the Advanced Statistical Analysis of the TRS 80 Model III personal computer (Radio Shack, Forth Worth, TX 76113) which could then project the concentrations of the "quality control" and patient samples. Means and standard deviations were also estimated from the above program.

Recovery

Recovery percentage of mCPP was estimated by comparing the peak heights of "quality control" (50 ug/L) samples to that of a known amount of injected mCPP.

Interference

For checking possible chromatographic interferences, the capacity factors, k' of some commonly used drugs were established.

RESULTS

Quantitation of mCPP concentration in plasma was achieved, firstly, by a simple three-step procedure: alkalinzation with sodium hydroxide,extraction with methylene chloride/isoamyl alcohol and back-extraction with diluted phosphoric acid, and secondly, by liquid chromatographic analyses using a reversed-phase column with a ternary mobile phase. Figure 2 shows the chromatograms of the extract of a "drug-free" plasma (Fig. 2A) with an unidentified peak Y, eluding at about 28 minutes (42 mL); a 25 ug/L plasma "standard" (Fig.2B); and a patient plasma with 17 ug/L of mCPP and 1402 ug/L of trazodone (Fig. 2C). Retention volumes of an unidentified peak, X, mCPP, IS and trazodone were 20.3, 22.7, 38.0 and 58.7 mL, respectively. Total analysis time for patient sample would be about 42 minutes. Peak height ratios were linearly correlated to mCPP concentrations up to 100 ug/L ($Y = 0.015 x + 0.052$. $r = 0.997$).

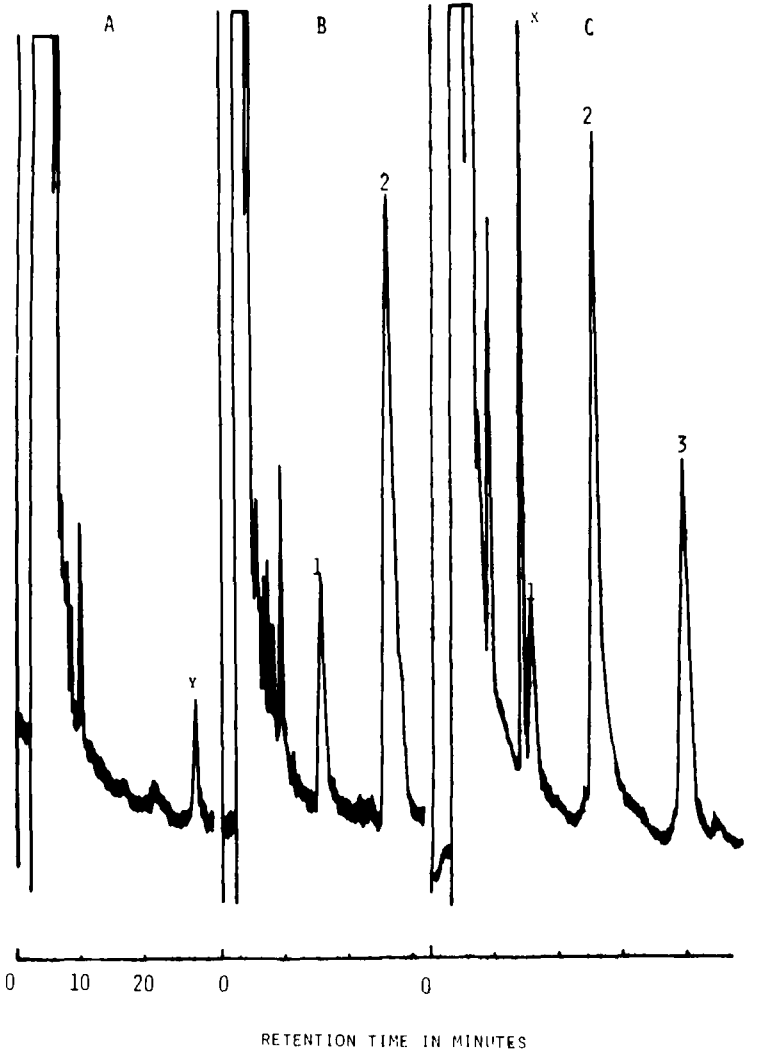

FIGURE 2: Chromatogram of plasma extracts: (A) "drug-free" plasma; (B) With a 25 ug/L plasma "standard" of mCPP (1) and IS (2); (C) A patient plasma with 17 ug/L of mCPP (1), IS (2), and 1402 ug/L of TRA (3) (Unidentified peaks X and Y)

TABLE 1

Precision Data of mCPP Assay

	CV	Mean, ug/L	n
a. Within-run	4.0%	49.2	6
b. Day-to-day	5.7%	49.6	26

TABLE 2

Capacity factors, k', of Some Common Drugs in Assay for mCPP

Drug	k'	Drug	k'
Acetaminophen	0.1	Chlordiazepoxide	a
Codeine	1.1	Perphenazine	a
Cimetidine	4.4	Pentobarbital	a
Trazodone Metab.(mCPP)	7.3	Flurazepam	a
Phenobarbital	8.2	7-OH-Amoxapine	a
Meperidine	9.0	8-OH-Amoxapine	a
Chloramphenicol	11.5	Phenytoin	a
I.S.	13.2	n-des.doxepin	a
Amitriptyline	a	Thioridazine	a
Desipramine	a	Secobarbital	a
Imipramine	a	Propoxyphene	a
Mianserin	a	Doxepin	a
Nortriptyline	a	Amoxapine	a
Chlorpromazine	a	Oxazepam	a
Trazodone	a(20.7)	Lorazepam	a

a = k' > 13.2

Sensitivity, defined as S/N ratio of 3, was 5 ug/L. Recovery of six "quality control" samples (50 ng/L) was estimated to be 50.0 $\pm$ 4.6%. Precision studies of "quality controls" showed acceptable coefficients of variation as outlined in Table 1. Capacity factors of some common drugs, as shown by Table 2, did not show any interference. Analyses of six patient plasma showed that the

following concentrations: trazodone (estimated from a previously established assay (1)) range = 576-1402 ug/L, and mean = 1024 ug/L; mCPP: range = 9-49 ug/L, and mean = 28 ug/L.

DISCUSSION

Trazodone, a new atypical antidepressant has been shown to metabolize through the hydrolytic cleavage to an active metabolite, mCPP and to possibly other yet to be identified metabolites (2,22-27). Recent studies showed previously unreported cardiotoxic side effects (9-13). However, in overdose cases, patients recovered with supportive therapies. In attempting to understand the clinical pharmacology of trazodone, such as the possible correlation of plasma concentration of trazodone and mCPP with response, the metabolism of trazodone in overdose patients, and the absorption and elimination of trazodone concommitant with food ingestion, quantitation of both trazodone and mCPP concentration in plasma would be helpful. As reviewed previously, published assays for determination of trazodone and mCPP were performed by using GC or LC-EC methods (2,25,32,33). Due to the unestablished clinical role of mCPP plasma concentration, initial effort was directed toward the development of a trazodone assay in plasma (1). The present study of mCPP is a continuation of that investigation, and on the development of LC assays for antidepressants and metabolites.

The polar nature of mCPP and the presence of an unidentified, X (metabolite) peak, closely eluding with mCPP as indicated by Suckow (2), placed stringent requirements for developing a reversed phase, "non-ion paired" LC analysis - specifically, using a high

carbon-load column (20%) and ternary mobile phase. Different from Suckow's procedure (2), the mobile phase used was an "ordinary", "non-ion paired" reversed-phase mobile phase, and detection was set at 254 nm at 0.001 AUFS, instead of a LC-EC, which is not yet readily available in most clinical laboratories. However, in order to establish a stable baseline at 0.001 AUFS, lengthy equilibration time of the chromatographic system would be needed, usually 1 hour. In effect, the present study established a simple LC assay utilizing readily available equipment and column, enhancing clinical monitoring of mCPP.

Assay Conditions

As described above, in choosing the chromatographic conditions, the strategy was to use a high carbon load column, thus relying on the increased interaction of mCPP with the packing. This approach was successfully used for the quantitation of 2-hydroxy desipramine (36), and 7- and 8-OH amoxapine (37) - (two metabolites of amoxapine - a new antidepressant - differing only in the OH position). In order to resolve a peak X and mCPP, preliminary systematic solvent scouting and temperature programming experiments with a 10 um particle column, uBondapak C-18, did not provide adequate retention and resolution. Attempts with a higher carbon load (20%) 5 um column and binary mobile phase - phosphate and ACN at various temperatures were not successful. Finally, a ternary mobile phase: phosphate: ACN: THF (90:5:5) provided the resolution.

Due to the high carbon load and the relatively "weaker" mobile phase (as compared to the mobile phase for TRA, phosphate = ACN

(72:28) (1), trazodone eluded at about 39 minutes. As shown by Figures 2A and 2B, an unknown peak Y eluded after the IS at about 28 minutes. However, the peak Y did not affect the peak height ratio as to result in unacceptable coefficients of variation, indicated by the data of the precision studies.

The present procedure was developed for mCPP, not for simultaneous quantitation of mCPP and trazodone. It would probably be possible to achieve this goal by adding another internal standard. In attempting to resolve mCPP and peak X, and eluding trazodone in a shorter time, it would be perhaps possible to employ gradient elution. However, this approach was not followed because for repeated clinical drug monitoring, gradient elution would require lengthy regeneration time.

In parallel with the optimization of the chromatographic conditions, systematic extraction studies were performed using the following organic solvents: ethyl acetate, methylene chloride with various percentages of isoamyl alcohol, petroleum ether, ether, isopropanol and ethylene glycol. Due to the polarity of mCPP, the data suggested methylene chloride/isoamyl alcohol (98:2) was the choice solvent. This mixture was chosen previously for extracting a polar metabolite of desipramine, 2-OH-desipramine (36). Similar to that study, the recovery of mCPP was also low, about 50% as compared to 66 to 68% for 2-OH-desipramine (36), and 80% for trazodone (1), 85 to 89% for maprotiline and 87% for both 7- and 8-OH amoxapine (37). This low percentage of recovery limited the sensitivity to only 5 ug/L. For other antidepressants, the limits were: 0.5 ug/L (desipramine) (38) to 5 ug/L (8 OH-amoxapine and

mianserin) (37). In retrospect, the analyses of six patients, as shown later on, indicated the limit of sensitivity at 5 ug/L was adequate. However, for pharmacokinetic studies or for monitoring patients with low doses, the sensitivity may be inadequate.

In assessing the stability of trazodone in-vitro, the previous study (1) showed that trazodone in frozen plasma would be stable up to three months. A similar approach was used for preparing mCPP working plasma stock solution and "quality control" samples. From the present date, it would indicate that mCPP was stable in frozen plasma. However, long term stability of mCPP in other media such as water or methanol was not assessed.

Patient Studies

With acceptable precision, the procedure was used to quantitate mCPP of six patients who had achieved steady state plasma concentrations of trazodone. The range of mCPP concentration was 9 to 49 ug/L, indicating that the designed calibration range (5-100 ug/L) for the assay was adequate. However, the sensitivity might limit this procedure from low dose pharmacokinetic studies. From this and a previous study, substantial plasma TRA concentration was identified and the ratio of mCPP/trazodone ranged from 1.5 to 6.3%. Thus, both the percentage and amount of mCPP were small and the clinical significance of this finding awaits future studies.

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