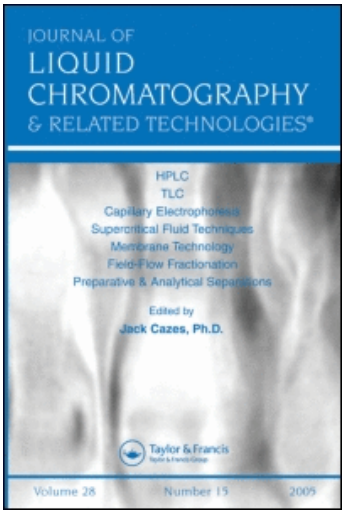

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DETERMINATION OF m-CHLOROPHENYL-PIPERAZINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC DETECTION

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ABSTRACT

m-Chlorophenylpiperazine (mCPP) and the internal standard, 1-(2-pyrimidinyl)piperazine (1PP) are extracted from base matrix using CN sorbent extraction columns. Chromatography and detection are performed using isocratic reverse-phase high performance liquid chromatography (HPLC) with a CN column and coulometric end-point detection. The standard curve was linear over the range 0-25ng/ml of plasma. The lower limit of quantitation is 0.2ng/ml of mCPP from 1ml of plasma. The reproducibility (CV) of the method over the range of the standard curve varied from 3-8.5%. The inter-assay CV was 5.9%. Recovery averaged 92.5 ± 8.6%. Plasma profiles of mCPP following varying doses of nefazodone HCl are given.

INTRODUCTION

Investigations have shown that m-chloro phenylpiperazine (mCPP, Fig 1) causes changes in serotonin (5-hydroxytryptamine, 5-HT) syntheses and turnover, these changes being consistent with

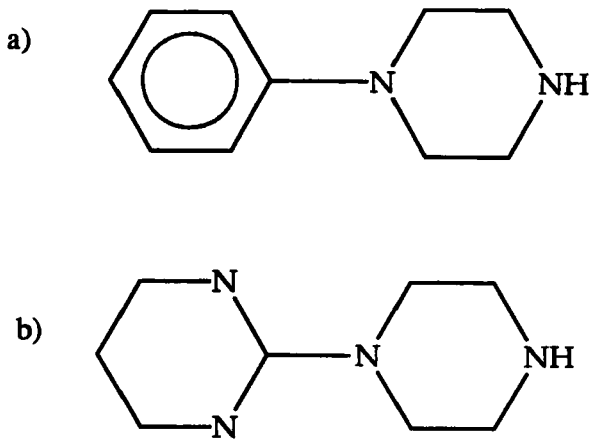

FIG 1

Structures of mCPP (a) and the internal standard 1PP (b).

post-synaptic 5-HT_{1A} angonism[1]. This compound has also been shown to cause classical, neuroendocrine, physiological and behavioural changes in animals and man which are reversed by 5-HT antagonist[2-4]. Recent studies in humans have investigated the neuroendocrine and behavioural effects of mCPP in various kinds of psychiatric disorders[5-6]. In both anxiety disorders and obsessive compulsive disorder, mCPP produced significant worsening of clinical symptomatology which in the case of obsessive symptoms correlated with plasma mCPP concentration.

The assessment of mCPP in plasma requires a highly sensitive & selective assay procedure to measure the very low levels present. Several methods to date have been reported. Caccia et al[7] used gas chromatography with electron-capture detection (GC-EC D)) following a derivatisation step. Others used high-performance liquid chromatography (HPLC) with UV[8,9] or electrochemical detection. Recently Suckow et al[10] reported a fairly sensitive (3ng/ml) HPLC technique utilizing UV detection,

however even this suffered from a lengthy extraction procedure. All these fail on two main counts, either they lack adequate sensitivity or the analysis time is exceptionally long. Hence an assay which is selective, sensitive and simple to carry out is required.

The simple method described here is based on solid phase sorbent extraction of mCPP from plasma followed by isocratic reversed-phase HPLC with coulometric detection. The method uses 1-(2-pyrimidinyl) piperazine (1PP, Fig 1) as an internal standard.

EXPERIMENTAL

Materials

mCPP was purchased from Aldrich Ltd (Gillingham, Dorset, UK). IPP, the internal standard was kindly donated by Bristol-Myers (Uxbridge, UK). The highest grade acetonitrile, methanol and potassium dihydrogen orthophosphate were purchased from BDH (Poole, Dorset, UK). Plasma for the preparation of standards was obtained from voluntary blood donors. All water was deionised and glass-distilled prior to use.

Cyanopropyl (CN) sorbent columns (Bond Elut) for extraction were purchased from Analytichem International (Habour City, CA 90710, USA). Stock standard solutions of both mCPP and 1PP, were prepared at concentrations of 100µg/ml in methanol. This was stored at 4°C and was stable for up to six months. Stock solutions for each compound were serially diluted in water for each assay run and finally made up to the required concentration in drug-free plasma.

CHROMATOGRAPHIC APPARATUS AND CONDITIONS

The HPLC system comprised of a Milton Roy Constametric 3000 pump (LDC Ltd, Stone, UK), a manual Rheodyne 7125 injection valve equipped with a 50µl loop, a 5µm particle size cyanopropyl analytical column (150mm X 4.6mm ID) protected by a 5µm particle

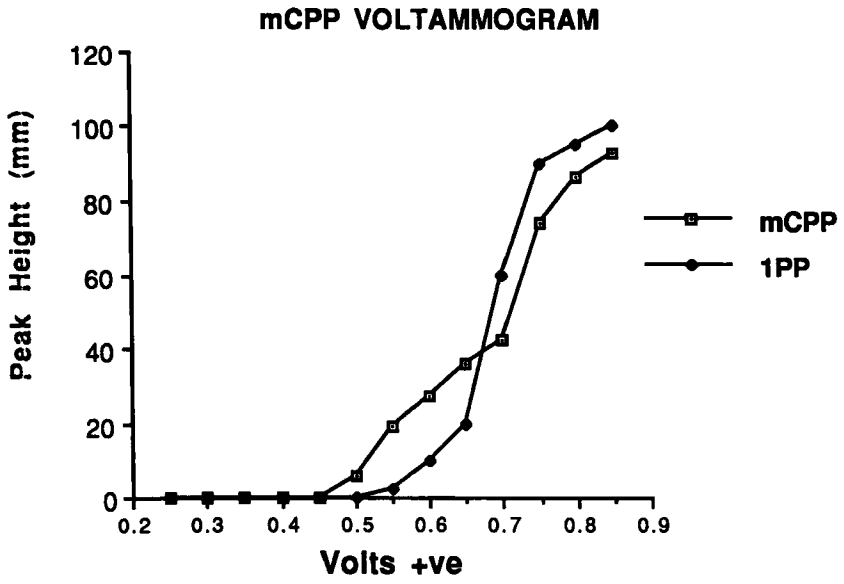

FIG. 2

Voltammogram of mCPP and 1PP, the internal standard, for detector 2 at different potentials. The voltammogram was determined when the guard cell and detector 1 were at zero potential.

size cyanopropyl guard column (Capital HPLC Ltd, Edinburgh, UK). THe detection system consisted of a Model 5100A coulometric detector and a Model 5020 guard cell (ESA, Bedford, MA, USA). The detector was linked to an LDC CI-4000 integrator (LDC, Stowe, UK).

The potentials for detectors 1 and 2 were selected after injection of fixed amounts of mCPP and the internal standard, 1PP over the range 0.2 - 0.85v for each detector. (Fig 2). The selected potentials for the guard cell and detectors 1 and 2 were 0.65, 0.75, and 0.55 volts respectively. The response time was set at 2 seconds.

The mobile phase consisted of 0.04M potassium phosphate buffer adjusted to pH 6.45 with 2M potassium hydroxide, HPLC-grade acetonitrile and methanol (600:250:150, v/v). The mobile phase was filtered and degassed prior to usage. The flow rate was 1.5ml/min.

The cyanopropyl columns were conditioned before the mobile phase was run through. This was done by initially flushing the columns with 50ml of distilled, deionised water, then with 0.005M sodium acetate buffer (pH 4.8) followed by acetonitrile in acetate buffer (2:3, v/v). Finally, the column was washed with 0.005M potassium phosphate (pH 4.8) before equilibration with the mobile phase.

Peak heights rather than peak areas in the chromatograms were normally measured. Concentrations of mCPP were assess by using the slope of the standard curve for peak-height ratios for the analyte and the internal standard.

PROCEDURES

Blood samples were collected into tubes containing lithium heparin as anticoagulant, centrifuged and the plasma separated and stored at -25°C until required for assay.

Standard curves were prepared fresh daily and consisted of five concentration points over the range 1-25ng/ml of mCPP in drug-free plasma. To each 1ml volume of standard or sample was added 100ng of the internal standard, 1PP (contained in 100µl) prior to column addition. Cyanopropyl Bond Elut sorbent columns (100mg) were initially conditioned with full column volumes of methanol and water respectively. The vacuum was diverted to keep the columns from drying out and the standards and samples were transferred to the columns. The vacuum was again applied allowing the materials to pass completely through. Each column was washed in turn with 2 column volumes of water. Columns were taken to full dryness under vacuum. The vacuum was again diverted, the manifold needles were wiped dry and a collection

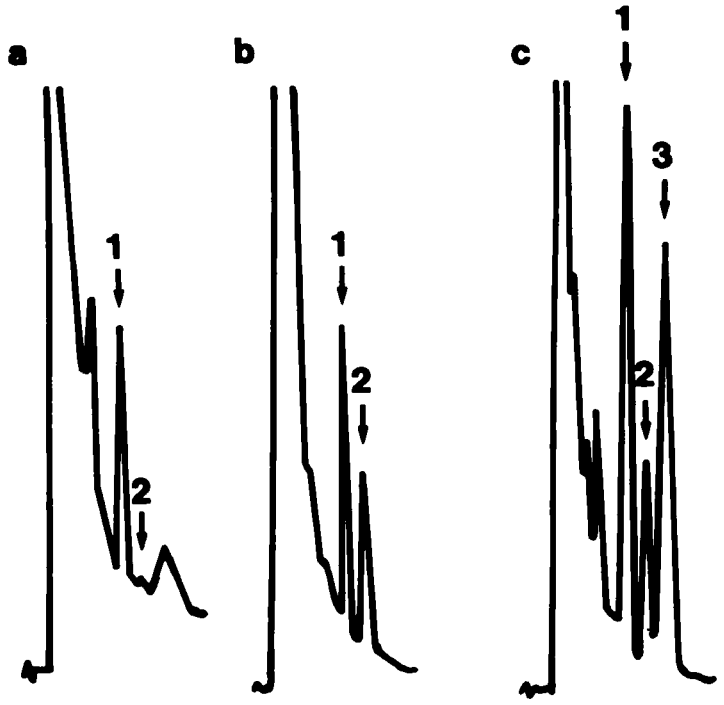

FIG 3

Chromatograms of (a) blank drug-free plasma, (b) drug-free plasma spiked with 10.0ng/ml mCPP and (c) sample from volunteer following oral administration of nefazodone HCl, mCPP peak equivalent to 5.2ng/ml. 1, 2 and 3 represents the internal standard, 1PP, mCPP and nefazodone respectively.

tray contained 10mm X 75mm glass tubes was inserted into the Vac Elut Manifold system.

Compounds were eluted with one column volume of methanol. The methanolic eluates were evaporated to dryness under vacuum at 40°C. Samples were reconstituted in mobile phase, vortex mixed and made ready for injection into the HPLC.

TABLE 1

INTRA-ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF mCPP IN HUMAN PLASMA (n=6)

Actual Value (ng/ml)	Observed Value (ng/ml)	Coefficient of Variation (%)*
2	2.11 ± 0.18	8.5
5	4.86 ± 0.3	6.2
10	10.02 ± 0.3	3.0

* The precision (coefficient of variation) of the method was calculated from results for pooled normal drug-free plasma spiked with known amounts of mCPP.

RESULTS

Resolution and sensitivity was determined by injection of an extracted plasma standard (Fig 3). The retention times of mCPP and the internal standard were 4.8 and 3.8 min respectively. The linearity of both the extraction procedure and the detector response (determined from the peak height) was verified over the anticipated range of the assay (0-25ng/ml). The linearity was determined by assaying pooled drug free plasma (which had been previously screened for extraneous peaks) spiked with known amounts of mCPP. A calibration curve was calculated for mCPP concentration and the peak-height ratio over the concentration range studied. The equation for the calibration curve was y=-0.736x +12.3 (r=0.997). Each point on the curve was calculated from the means of the intra-day assay variation data (Table I). The inter-assay coefficient of variation for a pooled plasma control was 5.9% ($\bar{x}$= 10.08 ± 0.59, n=10). The absolute recovery of mCPP from a drug free plasma spiked with 10ng/ml of mCPP was

TABLE 2

CHROMATOGRAPHIC MOBILITY OF SOME PSYCHOTROPIC DRUGS RELATIVE TO mCPP (50ng each injected)

Drug	Retention Relative to mCPP	Drug	Retention Relative to mCPP
mCPP	1	Amitriptyline	2.55
1PP	0.79	Fluphenazine	0.90
Nefazodone	1.33	Clomipramine	2.3
Gepirone	0.88	Mianserin	NR
Buspirone	1.08	Chlorpromazine	1.9
Valium	NR	Caffeine	NR
Haloperidol	1.03	Imipramine	1.51
Desimipramine	NR		

NR = No response after 20 minutes.

92 5 ± 8.64% (n=13). Sample extracts were stable for up to two weeks when stored out of light and at 4°C.

Chromatographic mobility data for several psychotropic drugs which could possibly interfere with the mCPP analysis are given in Table II. Plasma profiles of mCPP following placebo and three oral doses of nefazadone, of which mCPP is the major metabolite, in one male volunteer subject are shown in Fig 4.

DISCUSSION

Described here is a simple and highly selective HPLC assay procedure which utilises coulometric detection, solid-phase sorbent extraction and an internal standard, 1PP for monitoring extraction recovery and detector variation. The detection limit (ie peak height equal to three times baseline noise) was 0.07ng; thus allowing routine measurements of 0.2ng/ml in a 1ml plasma sample. It has been established that the ratio between the analytical recovery of mCPP and that of the internal standard submitted to the same operations was constant over a wide concentration range. Also, the detector response for both

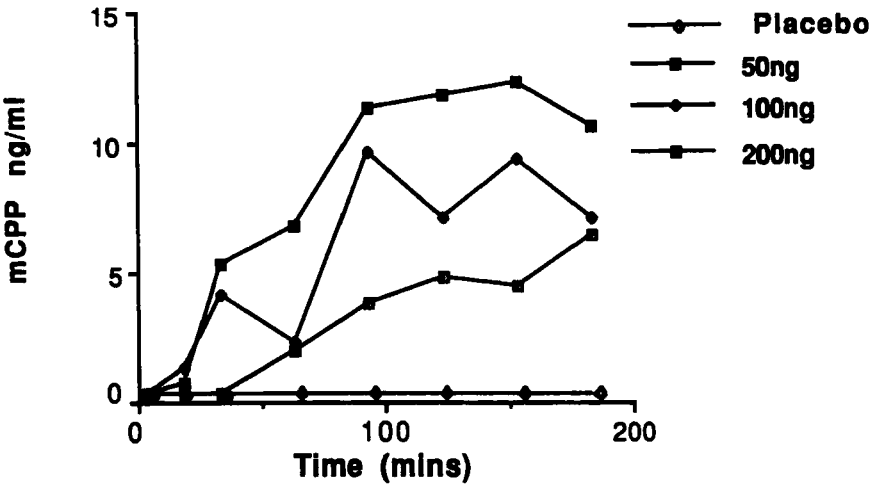

FIG 4

Plasma concentrations of mCPP in one male volunteer subject following oral administration of placebo and three doses of nefazodone on three separate occasions.

compounds was linear over the ranges tested. The requirements for an internal standard assay procedure were, therefore, satisfied.

Prior conditioning of the CN HPLC columns as described earlier, was found to be an important and essential pre-requisite for the avoidance of column blockade.

Extracted samples were stable for up to two weeks under the previously defined conditions and we found no sign of deteriorisation due to any procedural manipulations as can be seen from the chromatographic traces shown in Fig 3.

Samples from subjects taking haloperidol or fluphenazine would be expected to interfere with the analysis of mCPP. Other drugs tested would not be expected to interfere as can be seen

from Table II. However, caution is still necessary as some of the more polar metabolites of some of the tested compounds may well interfere eg the OH-metabolites. 1-(O-tylyl) piperazine 2HCl which was used as an internal standard by Suckow et al[11] was found to moreorless co-elute with mCPP under the defined chromatographic conditions.

The described procedure shows a number of improvements over previously reported methods. For example it is, quicker and simpler due to the very fast sorbent column extraction procedure. Twenty samples plus standards in replicate can be extracted and chromatographed in a single working day. It also has a much improved sensitivity (0.2ng/ml) as compared, for example, with Suckow et al who report a sensitivity of around 3ng/ml.

CONCLUSIONS

The method has been used successfully to analyse plasma concentrations of mCPP in one subject after various oral, doses given on different occasions, of nefazodone, a close relative of trazodone (see Fig 4). mCPP is the major metabolite produced by these compounds.

A novel technique using HPLC with coulometric detection has been described for the measurement of mCPP in plasma. It is fast, simple, reliable and relatively cheap to run. It is suitable for both routine clinical analysis and research purposes.

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