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Simultaneous determination of fluoxetine and its metabolite *p*-trifluoromethylphenol in human liver microsomes using a gas chromatographic–electron-capture detection procedure

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Abstract

An gas chromatography–electron-capture detection method has been developed for simultaneous determination of fluoxetine and *p*-trifluoromethylphenol (TFMP), an *O*-dealkylated metabolite of fluoxetine in human liver microsomes. Prior to the analysis, aliquots of alkalinized microsomal mixture were extracted with ethyl acetate solvent containing acetonitrile (10%, v/v) and the derivatizing reagent, pentafluorobenzenesulfonyl chloride (0.1%, v/v). The organ phase was retained and taken to dryness, the residue was reconstituted in methanol, and the aliquot of extracts was injected directly into a gas chromatograph equipped with an electron-capture detector. 2,4-Dichlorophenol was added to the initial incubation mixture and carried through the procedure as the internal standard. The method provided the mean recoveries of up to 103% for fluoxetine and 104% for TFMP. Acceptable relative standard deviations were found for both within-run and day-to-day assays. The practical limit of detection (signal-to-noise ratio=3) was 1.62 ng/ml for TFMP and 6.92 ng/ml for fluoxetine in human liver microsomes, and the limit of quantitation was 8.1 pg for TFMP and 34.6 pg for fluoxetine. The assay is rapid and sensitive and has been applied successfully to simultaneous quantification of fluoxetine and TFMP in human liver microsomes with different CYP2C19 genotypes.

Keywords: Fluoxetine; *p*-Trifluoromethylphenol

1. Introduction

Fluoxetine is a potent and selective serotonin reuptake inhibitor with antidepressant properties [1,2]. Fluoxetine is a derivative of phenylpropanolamine (Fig. 1), and it is extensively metabolized by the hepatic cytochrome P450 enzyme (CYP) and less than 2.5% of drug is recovered unchanged in the urine [3]. Fluoxetine currently known in human mainly undergoes *N*-demethylation to norfluoxetine and *O*-dealkylation to *p*-trifluoromethylphenol [4]. The disposition of fluoxetine and its stereoselective metabolism are associated with the polymorphic oxidation of debrisoquine, indicating that CYP2D6 is probably a major enzyme involved in its biotransformation [5]. However, von Moltke et al. [6] and recent our studies [7–10] found that polymorphic CYP2C19 play an important role in the *N*-demethylation of fluoxetine at a substrate concentration

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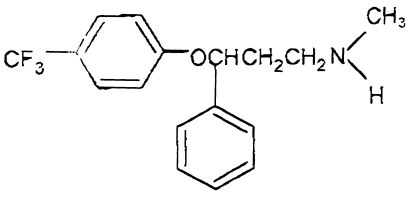
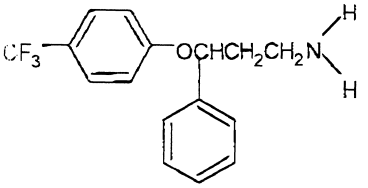


Fluoxetine



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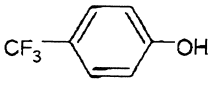
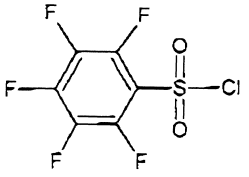


p-trifluoromethylphenol



Pentafluorobenzenesulfonyl chloride

Fig. 1. The chemical structures of fluoxetine, norfluoxetine, *p*-trifluoromethylphenol, and pentafluorobenzenesulfonyl chloride.

closed to the therapeutic level in vitro and in vivo and the contribution of CYP2C19 to fluoxetine *N*-demethylation among Chinese subjects be gene dose dependent. However, little is well known with regard to the isoforms of cytochromes P450 responsible for fluoxetine *O*-dealkylation. This could prove to be very important because metabolites are often either active therapeutically or they may contribute to the side effect profile of the parent drug and result in some dangerous drug–drug interactions [11–13]. In addition, metabolites may compete with other exogenous substrates for catabolic enzymes and result in marked changes in tissue and body fluid levels of these other drugs and/or their metabolites.

CYP2C19 is one of the cytochrome P450 isoforms concerning individual and ethnic variations of drug metabolism that have been studied most extensively in recent years. Based on the activity of CYP2C19 enzyme, individuals are phenotyped as extensive metabolizers (EMs) with normal enzyme activity and poor metabolizers (PMs) with a lower or deficient enzyme activity. CYP2C19-mediating *S*-mephenytoin 4′-hydroxylation show a genetically determined polymorphism that has marked interracial differences, with PM phenotype representing 2 to 5% of the Caucasian population but 13 to 23% of the Asian population [14]. Up to now, the normal allele and five defective alleles of *CYP2C19* have been designated as *CYP2C19*1* (wild-type), *CYP2C19*2* (*m1*), *CYP2C19*3* (*m2*), *CYP2C19*4* (*m3*), *CYP2C19*5* (*m4*), and *CYP2C19*6* (*m5*) [14,15]. Some studies found that the combinations of two genetic mutations (*m1* and *m2*) can account for 100% of Oriental poor metabolizers, whereas all of the genetic defects are found in Caucasians. Recently, two high-performance liquid chromatography methods have been developed in our laboratory for simultaneous quantification of fluoxetine and norfluoxetine in the human plasma and liver tissue [7,8]. We have found that there are some marked differences in the pharmacokinetic parameters of fluoxetine and norfluoxetine between the different genotyped individuals with respect to CYP2C19 [9] and contribution of CYP2C19 to fluoxetime *O*-dealkylation in human liver microsomes was gene dose dependent [16]. A gas chromatography–electron-capture detection (GC–ECD) procedure has been developed for the analysis of TFMP in biological sample [4]. But, to the best of our knowledge, fluoxetine and its *O*-dealkylated metabolite have never been quantitated simultaneously in human liver microsomes with different CYP2C19 genotypes. In order to quantitate accurately TFMP in liver microsomes, we describe in this paper a GC–ECD procedure that utilizes

pentafluorobenzenesulfonyl chloride as the derivatizing reagent. This reagent has the potential to be utilized for the extractive derivatization of amine-, phenol-containing drugs, tyrosyl peptides, nucleic acid pyrimidine bases, and proteins [4,17–20].

2. Experimental

2.1. Reagents

Fluoxetine hydrochloride was supplied by Research Biochemicals International (Natick, MA, USA). *p*-trifluoromethylphenol, 2,4-dichlorophenol (internal standard), and pentafluorobenzenesulfonyl chloride (PFBSC) were purchased from Aldrich (WI, USA). Omeprazole was a generous gift from Astra Hässle (Mölndal, Sweden). Coumarin, quinidine, triacetyloleandomycin (TAO), diethyldithiocarbamate (DDC), $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). Sulfaphenazole was a gift from CibaGeigy (Basel, Switzerland), and furafylline was kindly donated by Dr. W. Pfleiderer (Universität Konstanz, Wurzburg, Germany). Potassium hydrogencarbonate was supplied by Shanghai Chemical Center (Shanghai, China), and ethyl acetate from Tianjin (Tianjin, China). Acetonitrile was purchased from Tedia (Fairfield, OH, USA). All other chemicals were of analytical-reagent grade.

2.2. Human samples

Adult human liver tissues were collected from renal transplant donors and patients undergoing partial hepatectomy in our liver bank. The collection and utilization of human liver tissues for this study were approved by the Ethics Committee of Xiang-Ya School of Medicine, Central South University. Candidate patients for liver sample collection were those who did not suffer from acute or chronic hepatitis or cirrhosis, and took no medication known to induce or inhibit CYP activity. Portions of surgical liver ''waste tissues'' distant from disease-affected regions and appearing visually normal were collected. The collection approaches of liver tissues and its morphologic and biochemical characterization were described elsewhere [21]. Microsomes were prepared by differential centrifugation [22] and stored at −80 °C ready for use. Microsomal protein concentration was determined by the method of Lowry et al. [22].

Liver donors were genotyped for *CYP2C19* from whole blood or liver tissues according to the method of de Morais et al. [23]. Of the 34 liver donors, 18 liver microsomes were genotyped as homozygous EMs (*wt*/*wt*) with respect to CYP2C19, 13 heterozygous EMs (*wt*/*m1*), and three PMs with the m1 mutation (*m1*/*m1*). No *m2* allele was found.

2.3. Sample preparation and extraction

Fluoxetine metabolism in human liver microsomes in vitro was used in 0.1 mol/l potassium phosphate buffer (pH 7.4) containing 1 mg/ml of liver microsomal protein, the reduced NADP (NADPH)-generating system, and various concentrations of fluoxetine with or without inhibitors in a final volume of 500 μl. The enzyme reaction was initiated by adding 20 μl of three different of substrate concentrations (5, 25 and 100 μmol/l) in inhibitory experiment and ten concentrations of fluoxetine (1–200 μmol/l) in the enzymatic kinetics analysis of fluoxetine *O*-dealkylation. The NADPH-generating system consisting of 1 mmol/l NADP, 10 mmol/l glucose-6-phosphate, 2 I.U./ml glucose-6-phosphate dehydrogenase, and 10 mmol/l $MgCl_2$. After the incubation at 37 °C in a shaking water bath for 45 min, the reaction was stopped by cooling on ice and the adding of 100 μl acetonitrile. Preliminary experiments showed that the formation of TFMP was linear to an incubation time of over 60 min and a microsomal protein concentration of up to 2 mg/ml at 37 °C. Accordingly, the incubation time of 45 min and the microsomal protein concentration of 1 mg/ml were chosen in the present study.

After the termination of reaction, a fixed amount of internal standard (30 μmol/l 2,4-dichlorophenol in methanol) was added to the incubation mixture to assay fluoxetine and TFMP, and the solution was thoroughly shaken for 30 s. Following the procedure was established for derivatization with PFBSC as described by Urichuk et al. [4] with some minor modification. In brief, samples were basified preliminarily by adding excess potassium hydrogencarbonate (200 mg) and being briefly vortex-mixed. The

mixture was transferred to another clean glass tube to prevent the formation of an emulsion during the shaking and to avoid carrying excess potassium hydrogencarbonate through the whole procedure. Next, 4.5 ml of ethyl acetate containing acetonitrile (10%, v/v) and the derivatizing reagent, PFBSC (0.1%, v/v) were added to each sample. The samples were then vigorously shaken for 20 min in an YKH liquid-shaker (Jiangxi, China) and centrifuged for 10 min at 2000 *g*. The upper organic layer was retained and transferred to another screw-cap glass tube. The samples were eventually evaporated until dry under a gentle stream of nitrogen at 37 °C. The residue was reconstituted in 200 μl methanol and 2 μl aliquot was used for gas chromatography analysis.

2.4. Gas chromatography

The samples were analyzed using a gas chromatographic system consisting of a Hewlett-Packard (HP) 5890 series II gas chromatograph equipped with an ECD system and a HP-5 capillary column (cross-linked 5% PH NE siloxane, 15 m×0.53 mm, 1.5 μm film thickness). The carrier gas and make-up gas were ultra-pure nitrogen at flow-rates of 10 and 100 ml/min, respectively. The injector port and detector temperatures were 230 and 325 °C, respectively. The oven temperature was set initially at 80 °C which was maintained for 1 min and then increased at a rate of 10 °C/min up to a final temperature of 230 °C.

2.5. Calibration curves

Stock solutions of fluoxetine, TFMP, and 2,4-dichlorophenol were prepared in methanol at a concentration of 100 μmol/l. The stock solutions were stored at −20 °C.

To prepare standard curves, appropriate amounts of fluoxetine and TFMP were added to 500 μl of blank incubation buffer. The final calibration concentration ranges were 0, 1.62, 3.24, 8.1, 16.2, 32.4, 81, and 162 ng/ml for TFMP and 0, 3.46, 6.92, 17.3, 34.6, 69.2, 173, and 346 ng/ml for fluoxetine, respectively. These samples were then incubated and extracted according to the same procedure described above. Quantification was performed by calculating the peak-area ratio of each compound to the internal standard (2,4-dichlorophenol dissolved in methanol). In all experiments, the volumes of incubation mixture used in the calibration curves were the same as those used from the fluoxetine-treated samples. Calibration curves consisting of known, varying amounts of TFMP and a known, fixed amount of internal standard were included with each assay run.

3. Results and discussion

3.1. Selection of internal standard

2,4-Dichlorophenol has been used as an internal standard in previous report with GC–ECD to aid in quantitation of free *p*-trifluoromethylphenol in humans and rats treated with fluoxetine [4]. 2,4-Dichlorophenol was chosen as an internal standard because this compound could be well separated from fluoxetine and TFMP, and the percentage recovery of it from samples was similar to that of fluoxetine and TFMP under the same conditions used as well as no obvious interfering peaks were detectable in blank incubation buffer in human liver microsomes.

3.2. Chromatographic separation

Fig. 2 showed the chromatographic separations (A) obtained from extracted blank incubation buffer of human liver microsomes and spiked (B) with TFMP, 2,4-dichlorophenol, and fluoxetine as well as extracted incubation buffer (C) of human liver microsomes with fluoxetine and 2,4-dichlorophenol. The retention times for TFMP, 2,4-dichlorophenol and fluoxetine were 3.1, 4.5, and 10.1 min, respectively. The three peaks were completely resolved without obvious interference.

3.3. Validation study

The GC–ECD method described here enables simultaneous determination and quantification of fluoxetine and its metabolite TFMP in human liver microsomes. The calibration curves were found to be linear over the investigated range with coefficients of correlation (r^2)>0.9992. In addition, the calibration curves combined with recovery factors were utilized for quantitation. Hence, this method was validated for a wide concentration range from 1.62 to 162

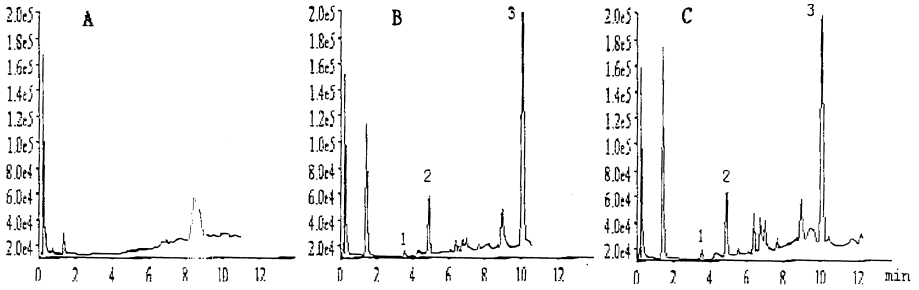


Fig. 2. Chromatogram (A) of extracted blank incubation buffer of human liver microsomes and (B) spiked with TFMP (0.2 μmol/l), 2,4-dichlorophenol (30 μmol/l), and fluoxetine (5 μmol/l), and (C) extracted incubation buffer of human liver microsomes with fluoxetine 5 μmol/l. Retention times: TFMP 3.1 min; 2,4-dichlorophenol 4.5 min; and fluoxetine, 10.1 min.

ng/ml for TFMP and 3.46 to 346 ng/ml for fluoxetine.

A good linear relationship was obtained in the range assay, 3.46–346 ng/ml for fluoxetine (r^2= 0.9995±0.001, n=5) and 1.62–162 ng/ml for TFMP (r^2=0.9992±0.002, n=5). The linear regression equations were y=10.814x+16.293 for fluoxetine and y=58.325x+38.007 for TFMP. The limit of quantification (LOQ) was determined using a signal to a noise ratio of 3:1. The LOQ was 1.62 ng/ml for TFMP and 6.92 ng/ml for fluoxetine. This sensitivity is comparable to or better than those reported previously [4], despite the small volume of sample used in our method.

We also investigated the precision of this method by calculating the relative standard deviation (RSD) of three different substrate concentrations measured on different days (n=10, inter-assay precision) and on the same day (n=6, intra-assay precision). As shown in Table 1, the RSD never exceeded 10.8% at any of the concentrations examined, indicating a good assay precision. The average recoveries of fluoxetine and TFMP ranged from 95 to 113% (Table 1), indicating a good recovery compared with previously described method [4].

Table 1
Intra- and inter-assay validation and relative recoveries of fluoxetine and TFMP

Concentration (ng/ml)	Intra-assay (%) (n=6)	Inter-assay (%) (n=10)	Recovery (%) (n=10) mean±SD
Fluoxetine			
3.46	7.1	10.2	108±11.2
6.92	10.4	6.8	106±10.2
346	8.3	9.5	96±8.8
TFMP			
1.62	6.9	7.4	95±11.8
3.24	7.8	5.8	113±8.4
162	9.7	10.7	104±9.6

3.4. Application

The method has been used to study the enzyme kinetics parameters for fluoxetine *O*-dealkylation in the homozygous EM liver microsomes, heterozygous EM liver microsomes, PM liver microsomes, and microsomal fractions of different human-expressed lymphoblast P450s in our experiment, with the same validation performance as that of previously published methods and improved extraction recoveries. Our study found that the kinetics of TFMP formation was best described by the two-enzyme and single-enzyme Michaelis–Menten equation for liver microsomes from CYP2C19 EMs and PMs, respectively. The mean intrinsic clearance (V_{max}/K_m) for the high- and low-affinity component was 25.2 and 3.8 μl/min/nmol P450 in the homozygous EM microsomes and 12.8 and 2.9 μl/min/nmol P450 in the heterozygous EM microsomes, respectively. At the substrate concentration of 5, 25, and 100 μmol/l, the mean microsomal levels of TFMP in the analysis of enzyme kinetic character of fluoxetine *O*-dealkylation in 11 human liver microsomes from EMs with respect to CYP2C19 were determined to be 372±56, 692±87, and 1037±159 pg/min/nmol P450 (Table 2). Representative chromatograms from incubation buffer using fluoxetine 5 μmol/l as a substrate are shown in Fig. 2C. Seven cytochrome P450s selective

Table 2
The formation rate of TFMP from fluoxetine *O*-dealkylation at three different substrate concentrations in 11 human liver microsomes from EMs with respect to CYP2C19

Fluoxetine concentration (μmol/l)	TFMP production (pg/min/nmol P450)
5	372±56
25	692±87
100	1037±159

inhibitors or chemical probes, including omeprazole (a CYP2C19 substrate, 100 μmol/l), quinidine (a CYP2D6 inhibitor, 10 μmol/l), sulfaphenazole (a CYP2C9 inhibitor, 20 μmol/l), furafylline (a CYP1A2 inhibitor, 25 μmol/l), diethyldithiolcarbamate (a CYP2E1 inhibitor, 20 μmol/l), triacetyloleandomycin (a CYP3A4 inhibitor, 50 μmol/l) and coumarin (a CYP2A6 substrate, 200 μmol/l) were proved to give no chromatographic interfering peaks with those of fluoxetine, 2,4-dichlorophenol, and TFMP [16]. In inhibition experiment, we found that omeprazole was a relatively weak inhibitor of the high-affinity site of TFMP formation, whereas its inhibitory effect on TFMP formation was greater at the low (5 μmol/l) substrate concentration than at the high (100 μmol/l) substrate concentration. In contrast, TAO had a strong inhibitory effect on this reaction at the high (100 μmol/l) substrate concentration than at the low (5 μmol/l) substrate concentration [16]. At the same time, our results still showed that TAO could produced different inhibitory effect on fluoxetine *O*-dealkylation in human liver microsomes with different CYP2C19 genotypes. At a low substrate concentration (5 μmol/l), TAO (50 μmol/l) had a relatively minor inhibitory effect (<47%) on TFMP formation in both the homozygous EM microsomes and heterozygous EM microsomes. However, the mean percentage inhibition by TAO was lower in the homozygous EM microsomes than in the heterozygous EM microsomes (35.3 versus 47.0%, $P<0.05$). With the increase of substrate concentration, the mean percentage inhibition of fluoxetine *O*-dealkylation increased to 47.4 and 59.3% at 25 μmol/l fluoxetine, and to 65.9 and 74.3% at 100 μmol/l fluoxetine in the homozygous and the heterozygous microsomes, respectively. In the PM microsomes, TAO almost abolished TFMP formation at all the three substrate concentrations (>90%) (Fig. 3).

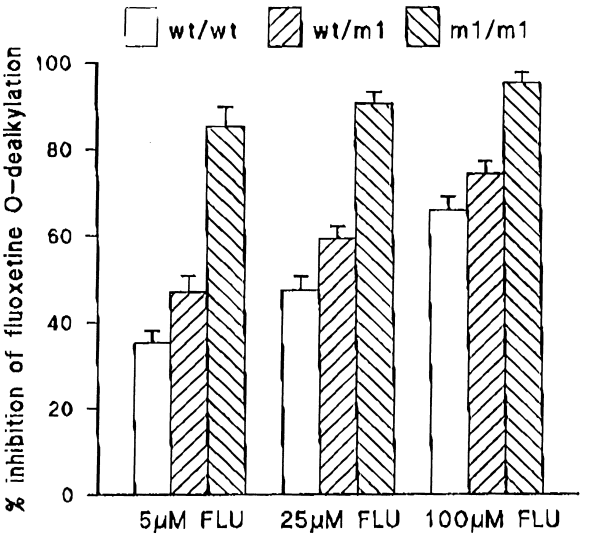


Fig. 3. Effect of TAO (50 μmol/l) on the formation of TFMP at different substrate concentrations (5, 25, and 100 μmol/l) in liver microsomes from different *CYP2C19* genotypes (three *wt/wt*, three *wt/m1*, and three *m1/m1*). The values are the mean inhibition percentage (±SD). FLU, Fluoxetine.

The procedure described above is a very useful method for the analysis and quantitation of TFMP in biological samples and has been well applied to the separation, detection, and simultaneous quantitation of fluoxetine and TFMP in liver tissues.

4. Conclusion

In summary, the use of PFBSC provides for the rapid extractive derivatization of fluoxetine, it also has the potential for use in the analysis of many other important drugs and endogenous substances. This method allows automated and simultaneous quantification of fluoxetine and TFMP over a wide concentration range in human liver microsomes. In addition, the extraction procedure is considered to be simple and convenient, and the samples were separated well without obvious interfering peaks. Accordingly, this method has been successfully used to determine the levels of fluoxetine and TFMP in human liver microsomes and study on the identifica-

tion of CYP isoforms responsible for fluoxetine *O*-dealkylation.

Acknowledgements

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