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Note

New high-performance liquid chromatographic method for fluphenazine and metabolites in human plasma

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Phenothiazines have traditionally been of interest to chromatographers because of the clinical importance of their quantification and because their complex structures make them challenging to chromatograph well. The application of high-performance liquid chromatography (HPLC) to the clinical or research evaluation of phenothiazine levels is limited by the resolution of the method. This resolution is of especial importance in the study of the contribution of the structurally similar phenothiazine metabolites to both the toxic and therapeutic effects of these drugs.

Drug metabolism is essentially the manipulation of the polarity of compounds in order to influence their activity and distribution and thereby regulate their actions and elimination. Reversed-phase HPLC is the method of choice for the study of drug metabolism as these separations are primarily based on polarity and hydrophilicity of compounds, which are the same parameters manipulated in drug metabolism. In order to utilize the resolution of closely related compounds which is provided by reversed-phase chromatography, we investigated methods for the reversed-phase chromatography of phenothiazines and related compounds [1–11]. We have developed an HPLC method resolving fluphenazine (FPZ) and its metabolites, with selectivity and high sensitivity provided by the use of electrochemical detection (ED). It offers not only improved resolution but also generates clinically applicable information on the levels of parent drug and metabolites. The method may also be used with all the other structurally related phenothiazines. Further, the volatile buffer system is compatible with post-column analyses.

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EXPERIMENTAL

Instruments and materials

A Model 510 pump, Model 710 autoinjector, Model 481 variable-wavelength absorbance detector (all from Waters Assoc., Milford, MA, U.S.A.), an LC-4B electrochemical detector and column heating block (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and a strip chart integrator (Model 3380, Hewlett-Packard, Palo Alto, CA, U.S.A.) were used. Reversed-phase HPLC of phenothiazines was performed on a C_{18} column (Waters μBondapak, 300×3.9 mm; 10 μm particle diameter) and guard column packed with pellicular C_{18} (Waters Corasil C_{18}, 40 μm particle diameter) with detection at a glassy carbon electrode at a potential of +850 mV. FPZ and its metabolites (FPZ-S-oxide, FPZ-7-OH, FPZ-8-OH, and FPZ-N-oxide) were gifts of E.R. Squibb and Sons. Solvents (water, Fisher, St. Louis, MO, U.S.A.; hexane, acetonitrile and methanol, American Burdick and Jackson, St. Louis, MO, U.S.A.) were all HPLC grade. Isoamyl alcohol (AR) was from Mallinckrodt (St. Louis, MO, U.S.A.). Formic acid and sodium octane sulfonic acid (both 98%, ACS) and ammonium carbonate and tridecylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.); phosphoric acid, dimethyl-, diethyl-, trimethyl- and triethylamines and tetrabutylammonium phosphate and di-N-butylamine were purchased from Sigma (St. Louis, MO, U.S.A.). In all experiments the colum was maintained at 40°C with the column heating block. At the end of each day's runs, the column was washed in water and stored in 70% methanol. All glassware was silanized prior to use.

Plasma extraction

To determine the efficiency of recovery, naive pooled plasma was spiked with different concentrations of FPZ and metabolites. The UV absorbance was integrated, and recovery calculated from comparisons of peak areas. For study of metabolites in blood, informed consent was obtained from inpatients prescribed oral fluphenazine. Blood (ca. 5 ml) was drawn into a vacuum tube containing sodium citrate and centrifuged at 1000 *g* for 15 min. Plasma (1 ml) was pipetted into a glass screw-top test tube and 1 ml of potassium carbonate was added and vortexed. Organic solvent (6 ml) was then added and the test tube shaken for 10 min. The organic layer (5 ml) was drawn out and transferred to a second tube and evaporated to dryness, either under a stream of nitrogen or in a vacuum centrifuge (Speed-Vac; Savant Instruments, Farmingdale, NY, U.S.A.). The residue was reconstituted in 1 ml methanol and 10–50 μl were injected into the HPLC system. Extractions of plasma itself showed no evidence of endogenous interfering substances (see Fig. 2A). Recoveries of FPZ and metabolites with a single isoamyl alcohol extraction are shown in Table I, as demonstrated with plasma samples spiked with known quantities of FPZ and metabolites.

RESULTS

Plasma extraction

Hexane was not found to be usable for extractions because, although FPZ was recovered with an efficiency of 90% with hexane extraction, all the metabolites

TABLE I

RETENTION TIMES AND RECOVERIES OF FLUPHENAZINE AND METABOLITES FROM PLASMA

Recoveries were calculated from three separate plasma samples spiked with known amounts of drug and metabolites. Variability of recovery was less than 5% among the three determinations for parent drug and any of the metabolites. Retention times vary less than 2%.

Compound	Retention time (min)	Recovery (%)
FPZ-S-oxide	3.5	102
FPZ-7-OH	6.1	94
FPZ-8-OH	8.0	103
FPZ	12.7	94
FPZ-N-oxide	14.2	101

were recovered at less than 20% efficiency. Hexane with 1 or 2% isoamyl alcohol gave essentially similar results. Extraction of the alkalinized plasma with 100% isoamyl alcohol resulted in excellent recoveries of FPZ and metabolites (Table I).

Chromatography

The initial method employed utilized 0.01 *M* ammonium carbonate in 25% methanol (pH 7.2) at a flow-rate of 1 ml/min. This was found to be unsatisfactory because of poor peak shape and basic pH which rapidly leads to column degradation and unacceptably short column working life. A significant improvement was made by using 0.02 *M* phosphoric acid in 45% methanol with 5 m*M* tridecylamine. The phosphoric acid kept the pH low (1.9) to improve ion pairing, but tridecylamine is not very soluble and regularly would precipitate in injection valves, columns and tubing, resulting in large pressure fluctuations and inconsistent chromatography.

The other ion-pairing reagents tested, including dimethyl-, diethyl-, trimethyl- and triethylamines and tetrabutylammonium phosphate and sodium octane sulfonic acid, did not significantly improve peak shape at concentrations up to 15 m*M*. However, di-N-butylamine has the requisite characteristics (a strongly basic amine with relatively long non-polar aliphatic groups attached) for successful chromatography of the phenothiazines.

Concentrations of both methanol and acetonitrile ranging from 30 to 60% were investigated for their actions on both retention and on peak shape. Methanol provided superior peak shape and also gave a reasonable retention time for FPZ and metabolites at 50% concentration.

In order to perform post-column analyses, a readily removable buffer salt was sought. Formic acid at 0.15 *M* was found to provide peak shapes comparable or superior to those obtained with phosphoric acid (Fig. 1). Lower concentrations (0.05 and 0.1 *M*) did not appear to maintain a pH low enough to facilitate ion pairing. The final mobile phase selected was 0.15 *M* formic acid and 0.01 *M* dibutylamine in 50% methanol, pH 3.2, at a flow-rate of 2 ml/min. This buffer provided baseline resolution of FPZ-S-oxide, FPZ-7-OH, FPZ-8-OH, FPZ and FPZ-N-oxide, with retention times of 3.5, 6.1, 8.0, 12.7 and 14.2 min, respectively. The

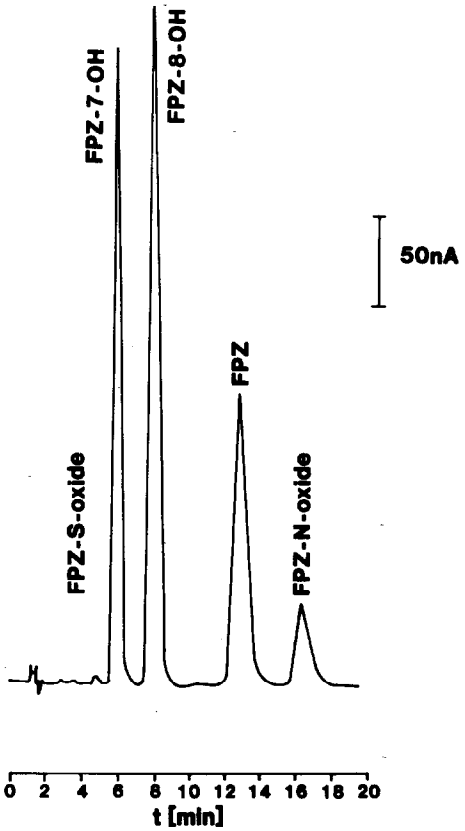

Fig. 1. Representative chromatogram of standard injection of FPZ and metabolites extracted from spiked plasma. Mobile phase is 0.15 *M* formic acid and 0.01 *M* dibutylamine in 50% methanol, pH 3.2. Flow-rate is 2 ml/min and the column is maintained at 40°C. Potential at the glassy carbon electrode is set at +850 mV versus an Ag/AgCl reference electrode.

retention of the sulfoxide was determined using UV detection, as it was not readily detected with ED. Chlorpromazine eluted at 9.2 min (not shown).

The detection limit (signal-to-noise ratio > 2) of the electrochemical method for standard injections was 300 fmol. In comparison, the detection limit was not as low with UV absorbance at 254 nm. However, FPZ-S-oxide was more easily detected with UV than ED, as has been reported by Brooks and DiDonato [1] for chlorprothixene and its sulfoxide. The potential setting of +850 mV was found to be optimal for ED of these compounds.

This method has been applied to the determination of FPZ and its metabolites in the blood of patients taking FPZ. Two representative chromatograms of patient plasmas are shown. Plasma from patient 1 appears to contain FPZ, the 7- and 8-hydroxyl and N-oxide metabolites (Fig. 2B). In contrast, patient 2 (Fig. 2C) has a much higher plasma level of FPZ and does not appear to metabolize it significantly. Of the two, patient 2 demonstrated more sedation on this dose of FPZ.

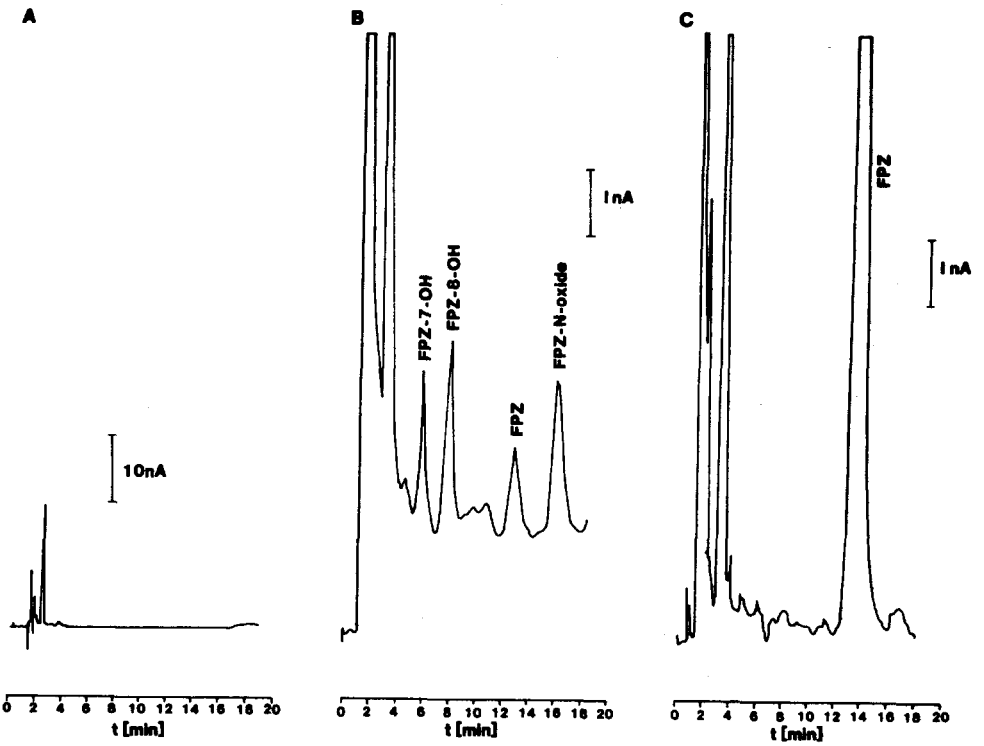

Fig. 2. Chromatograms of plasma alkalinized, extracted and chromatographed as described in text. (A) Chromatogram of non-drug plasma. (B and C) Chromatograms of plasma extracts from two patients. Patients were administered the same dosage (Prolixin hydrochloride, 5 mg three times a day, orally) for approximately five days. Plasma was drawn 1 h after the morning oral dose. Patient 1 (B) was also taking amantadine (100 mg twice a day) and theophylline (350 mg twice a day). Patient 2 (C) was also taking alprazolam (0.5 mg) and benztropine (1 mg) daily.

DISCUSSION

Reversed-phase HPLC of the phenothiazines has been limited by the phenothiazines' chemical structure, a tricyclic ring with both polar and non-polar substituents. FPZ and related phenothiazines are especially difficult to chromatograph well, as the piperazine side-chain of these compounds participates strongly in the separation mechanism as well, presumably by hydrophilic interactions with exposed silanol sites on the reversed-phase column. This mixed mechanism of separation generally results in split peaks, poor peak shape and excessive tailing and asymmetry, especially of later-eluting peaks. These technical difficulties have led some investigators to the use of normal-phase methods for the separation of phenothiazines, including the use of both bare silica [10] and cyanopropyl stationary phase in the normal-phase mode [5].

In the reversed-phase mode, one may either attempt to suppress ionization of the phenothiazine by operating at the highest pH compatible with column stability or lower the pH and ion pair the ionized groups with alkylamines. In the latter case, the lowest attainable pH is desired, to promote ionization and ion

pairing. Lowest possible concentrations of salts are also desired, so as not to interfere with the formation of the required ion pairs.

The present method offers improved resolution, lower detection limit and greater simplicity of use than other HPLC methods in the literature [1–11]. In addition, the solvent components, including the ion-pairing reagent used to suppress peak tailing, form azetropes [water–formic acid (22.5:77.5) and water–dibutylamine (50.5:49.5)], which are readily removed under vacuum and do not require neutralization nor desalting prior to subsequent post-column analyses. Such analyses may extend the sensitivity and selectivity of the HPLC method.

This study demonstrates the applicability of this method for the identification of specific metabolites of FPZ, many of which have been reported to be present in plasma or tissue after administration of FPZ [2,11–14]. Further studies now in progress will provide more definitive identification of the chemical nature of the metabolites than is possible by HPLC retention time alone.

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