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Note

Assay of plasma thioridazine and metabolites by high-performance liquid chromatography with amperometric detection

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The clinical utility of monitoring plasma levels of phenothiazines and their metabolites is not yet firmly established, although past work suggests that there is a relationship between plasma level and clinical effects [1]. Adequate assay methods are necessary to further elucidate this relationship and to investigate the pharmacokinetics of this important class of antipsychotic agents. Thioridazine (Mellaril) is a particularly interesting antipsychotic agent in this regard because it is commonly used clinically and its clinical effects may be highly dependent on the relative proportions of its individual metabolites in blood and tissue [2]. Since the introduction of thioridazine in the late 1950s, several analytical procedures have been described for its determination in human plasma [3—5]. Most frequently, a gas or liquid chromatographic method has been employed. Among the liquid chromatographic assays, both adsorption (normal-phase) [6] and reversed-phase [7] techniques have been used. Detection of the eluted compounds has typically been accomplished with ultraviolet (UV) absorbance methods [8], although electrochemical detection is becoming more popular for the phenothiazine class of drugs [9, 10]. This report describes a reversed-phase high-performance liquid chromatographic (HPLC) system coupled to an amperometric detector for the determination of thioridazine and three pharmacologically active metabolites in human plasma. The assay described provides several advantages over existing methods: (a) increased chromatographic resolution and efficiency, (b) increased detector selectivity and sensitivity, and (c) better sample recovery.

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EXPERIMENTAL

Apparatus

The HPLC system consisted of an Altex Model 110A pump, an Altex Model 210 injector equipped with a 500-μl sampling loop (all from Rainin Instruments, Woburn, MA U.S.A.), and a cyano-bonded reversed-phase column (30 cm × 3.9 mm I.D. Waters Assoc., Milford, MA, U.S.A.). An amperometric detector was employed (Model LC-4) using a glassy carbon working electrode maintained at 0.9 V relative to a silver/silver chloride reference electrode (all from Bioanalytical Systems, West Lafayette, IN, U.S.A.). The detector signals were plotted on a strip-chart recorder (10-mV full-scale pen deflection; Houston Instruments, Austin, TX, U.S.A.). Other columns used during methods development (μBondapak C_{18}, μBondapak Phenyl, and μPorasil; all with 10-μm particle size) were also purchased from Waters Assoc.

Materials

All solutions were made with ultrapure water, prepared by a reverse osmosis, deionization, and charcoal filtration system (Hydro Service, Weymouth, MA, U.S.A.) Tetrahydrofuran (THF), hexane, and diethyl ether were all HPLC grade (Fisher Scientific, Medford, MA, U.S.A.). All buffer salts and other reagents were analytical grade or better.

Authentic standard compounds (thioridazine·HCl, mesoridazine·besylate, sulforidazine, northioridazine, thioridazine-5-sulfoxide, and thioridazine-2,5-disulfoxide) were all generously provided by Sandoz Pharmaceuticals (Basel, Switzerland). Chlorpromazine·HCl was donated by Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.).

No special surface treatment was found to be necessary for the Pyrex glassware. All tubes and caps used in the sample preparation and storage were polypropylene (Elkay Products, Shrewsbury, MA, U.S.A.).

Chromatography

The mobile phase consisted of 70% (v/v) 20 m*M* monosodium phosphate containing 0.1 m*M* disodium ethylenediaminetetraacetic acid (Na_2EDTA), prefiltered through a 0.45-μm pore cellulose microfilter (Millipore, Bedford, MA, U.S.A.), and 30% THF. This mixture was adjusted to pH 4.0 and degassed by ultrasonication for 10 min. The chromatographic flow-rate was maintained at 1.5 ml/min for all separations.

Sample preparation

Plasma samples were prepared from whole blood drawn into heparinized tubes (Venoject, Kimble-Terumo, Elkton, MD, U.S.A.). Following a low-speed (2000 *g*), refrigerated (4°C) centrifugation, the clear plasma was transferred to, and stored in capped polypropylene tubes at −20° C until assay.

For the phenothiazine extraction, 1.0 ml of plasma was pipetted into a 12 × 75 mm polypropylene tube containing 100 μl of 10 *M* sodium hydroxide and 100 μl (1.0 μg) of authentic chlorpromazine in water as the internal standard. Following brief vortex-mixing, 2.5 ml of freshly prepared diethyl ether—hexane (3:1, v/v) was added. This mixture was vortexed vigorously for

1 min and then centrifuged at 6000 *g* for 5 min. The sample tubes were then placed on crushed dry ice. When the lower, aqueous phase was frozen, the organic phase was decanted into a 12-ml capacity polypropylene tube containing 2.5 ml of 0.1 *M* citric acid. The tubes were capped, vortexed for 30 sec, and then centrifuged at 6000 *g* for 5 min. The organic phase, now devoid of phenothiazines, was aspirated to waste. To the aqueous phase, 250 μl of 10 *M* sodium hydroxide and 0.9 g of sodium chloride were added, followed by 1.0 ml of hexane. The samples were agitated on a rotary inversion mixer for 15 min, after which time they were centrifuged for 5 min at 6000 *g*. Samples were then placed on crushed dry ice, as before. The hexane phase, containing the phenothiazines, was decanted from the frozen aqueous layer into a 12 × 75 mm polypropylene tube containing 1.0 ml of 0.1 *M* citric acid. This new mixture was vortexed and centrifuged as before. Following aspiration of the hexane phase to waste, 500 μl of the citrate phase was injected into the HPLC system.

Standard solutions of authentic phenothiazines in plasma and water were always assayed in parallel with unknown samples. Concentrations of thioridazine, mesoridazine, sulforidazine, and northioridazine of 50, 100, 500, and 1000 ng/ml were found to be appropriate for comparison to clinical samples.

RESULTS AND DISCUSSION

Chromatography

Several chromatographic schemes were tried without success before discovery of the system discussed in Experimental. Our first attempt, using a modification of a previously reported normal-phase method [6], employed a mobile phase of isooctane—water-saturated methylene chloride—methanol (9:1:1, v/v/v) containing 200 μl/l diethylamine. The column used was a 30 cm × 3.9 mm I.D. stainless-steel μPorasil column. The column provided excellent resolution of the drug standards thioridazine, northioridazine, sulforidazine, mesoridazine, thioridazine-5-sulfoxide, and thioridazine-2,5-disulfoxide. Due to the UV-absorbing characteristics of our mobile phase, the UV detector was tuned to a wavelength of 285 nm, instead of 254 nm as employed by Kilts et al. [6]. Although the detector provided adequate sensitivity for clinical samples, a large interfering peak, present only in patient blood specimens and coeluting with mesoridazine, yielded erroneously high mesoridazine values. This interfering signal may represent an additional, ring-oxidized metabolite* of thioridazine amplified preferentially at the selected detector wavelength.

The UV detector was abandoned in favor of the more sensitive and selective amperometric detector. Since electrochemical detectors rely on electrically conductive mobile phases, a suitable reversed-phase system was sought. In early trials we used previously reported methods incorporating acidic buffers, polar organic solvents, and trace organic amine modifiers in the mobile phases. Octadecylsilica (ODS or C_{18}) and phenyl-bonded silica (μBondapak C_{18} and

*Of the many theoretically possible thioridazine metabolites, this is most likely a ring-sulfoxide or sulfone because it is not detected electrochemically; such detection requires a reduced ring-sulfur atom in the oxidation process at the carbon electrode.

μBondapak Phenyl, respectively) stainless-steel columns were employed with these mobile phases. The chromatographic results were disappointing with each mobile phase combination tried. Individually, each of the four compounds tested (mesoridazine, sulforidazine, thioridazine, and northioridazine) gave usable peaks, but, unfortunately, the pairs mesoridazine—sulforidazine and thioridazine—northioridazine coeluted exactly.

ODS columns have been found to exhibit excellent resolving power with moderately polar organic molecules, such as the monoamine neurotransmitters and their metabolites. The phenothiazines, as well as other relatively large drug molecules, are much less polar, however, so that the ODS column characteristics are less appropriate. Additionally, ODS colums usually require a small amount of organic amine modifier in the mobile phase to prevent peak tailing. These organic amines react with and passivate glassy carbon electrode surfaces, thereby causing large losses of sensitivity over time [11].

Recently, other bonded phases have become available. These columns exhibit diverse and unique chemical characteristics. Wallace et al. [12] reported an assay for the non-antipsychotic phenothiazine drug promethazine using a normal-phase column (MicroPak CN-10) with cyanopropyl as the phase bonded to silica and a mobile phase of acetonitrile—20 m*M* KH_2PO_4 (45 : 55). Thus a cyano-bonded column seemed a promising choice.

A Waters Assoc. reversed-phase cyano-bonded column was tried with the mobile phase used by Wallace et al. [12]. Although mesoridazine gave a sharp,

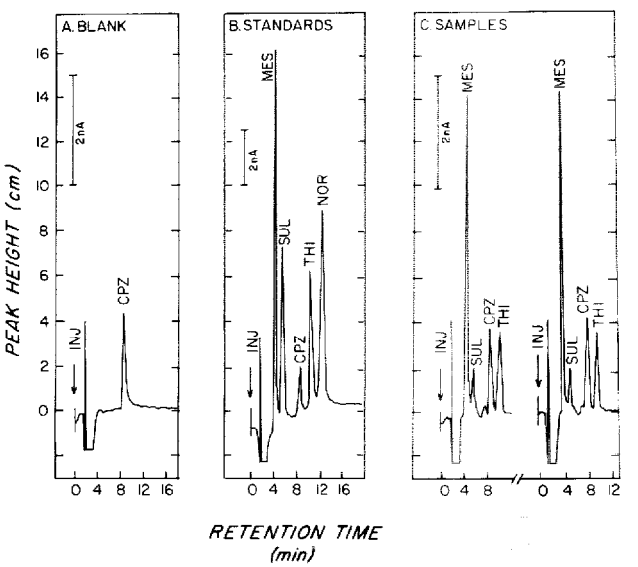

Fig. 1. Sample chromatograms. (A) Blank, extracted human plasma containing 1000 ng/ml chlorpromazine (CPZ) as the internal standard. The initial biphasic solvent front is due to the citric acid in the injection vehicle. (B) Normal human plasma extract, containing authentic standards (1000 ng/ml) of mesoridazine (MES), sulforidazine (SUL), chlorpromazine (CPZ), thioridazine (THI), and northioridazine (NOR). Note that sensitivity is 50% that in examples A and C. (C) Duplicate assays of plasma extracts from a patient receiving thioridazine (200 mg per day, orally). Blood was drawn at 12 h after the last daily dose following two weeks of this treatment. Note typical absence of northioridazine at this detector sensitivity.

early-eluting peak, thioridazine eluted much later, as a broad asymmetric hump. Methanol and butanol, at various pH values, were tried unsuccessfully as solvent modifiers. THF was then tried, with better results.

A final mobile phase composed of 20 m*M* monosodium phosphate containing 0.1 m*M* Na_2EDTA—THF, pH 4.0 (70:30, v/v) was chosen. This system resolved mesoridazine, sulforidazine, thioridazine, and northioridazine in less than 14 min at a flow-rate of 1.5 ml/min (see Fig. 1). Virtually symmetrical peaks were obtained without any organic amine modifier in the mobile phase. The elution order indicated mixed reversed- and normal-phase chromatographic behavior of the system. The sulfoxyl metabolites of thioridazine, mesoridazine, and sulforidazine eluted before the less polar parent compound, as is expected in a reversed-phase system. Thioridazine, however, eluted before its desmethylated and thus more polar metabolite northioridazine.

The ring-sulfoxyl metabolites of thioridazine are not detectable by amperometry, presumably because a reduced ring-sulfur atom is necessary for oxidation at the glassy carbon electrode. Since the ring-sulfoxyl metabolites of thioridazine are believed not to be active as antipsychotic agents [2], the added selectivity of the present assay, which excludes them, is welcome. Furthermore, amperometric detectors, which can reliably detect less than 1 ng of a phenothiazine injected on a column, give the present method other potential clinical applications since highly potent phenothiazines, such as fluphenazine (Prolixin), are typically present in blood at levels below 1 ng/ml [13].

Sample preparation

A back-extraction method was found to be necessary for the phenothiazines due to the poor recovery experienced when evaporation—concentration steps were used. Although some previous authors did not report this problem [14, 15], we found that this class of drugs adhered strongly to the sample tubes following solvent evaporation under a gentle stream of nitrogen gas. Recovery of the phenothiazines did not exceed 20% following reconstitution of the residue in various buffers or methanol—buffer mixtures. This effect persisted even when tubes composed of polypropylene, polyethylene, PTFE, stainless steel, Pyrex, or flint glass were used for the evaporation—reconstitution step. Coating the inside walls of sample tubes with an organosilane surface-treating agent (Prosil-28, PCR Research Chemicals, Gainesville, FL, U.S.A.) had little or no effect on the recovery of thioridazine.

The use of large injection volumes eliminated the need to concentrate samples. Little or no loss of resolution occurred, nor was any interference from the void volume observed, with injected samples volumes of up to 500 μl. Presumably, even larger volumes can be used, if necessary.

Recovery, precision and linearity

The recovery of the water-based standards averaged 76% for standards at 50, 100, 500, 1000, 5000, and 10,000 ng/ml (one sample at each concentration). The plasma-based standards, however, exhibited somewhat lower recoveries, averaging a high of 71% for thioridazine, but 62% for chlorpromazine, and only 39% for mesoridazine (one sample each at 10, 50, and 100 ng/ml), presumably due to lipophilic interactions between drugs and plasma constitu-

ents. Nevertheless, these values were reliable and the use of peak height ratios, relative to the internal standard, chlorpromazine, adequately corrected for the recovery losses in the plasma samples. This conclusion is supported by the linearity of the ratio of peak heights for thioridazine and its metabolites to chlorpromazine across a wide range of concentrations likely to be encountered in clinical specimens.

Both water- and plasma-based standards yielded highly linear relationships based on peak height or on the ratio of peaks for thioridazine or mesoridazine to that of the internal standard, chlorpromazine, in the range 10—1000 ng/ml ($r > 0.99$). This range can be extended downward to the limit of detection of thioridazine and other phenothiazines, about 0.1 ng per injection. Above 1000 ng, however, the detector signals began to level off, presumably due to the configuration of the working and reference electrode pair.

In a study of precision of the method, plasma samples from each of eight patients receiving thioridazine were assayed in duplicate. Mean coefficients of variation for thioridazine, sulforidazine, and mesoridazine were 7.97% 8.12%, and 7.89%, for mean concentrations of 250.3, 81.44 and 258.2 ng/ml, respectively.

CONCLUSIONS

Reversed-phase HPLC with electrochemical detection is a powerful and useful tool in the determination of plasma thioridazine, its metabolites, and other phenothiazines. The use of a cyano-bonded silica column with tetrahydrofuran as the organic modifier in the mobile-phase buffer allowed clear resolution of thioridazine and three of its pharmacologically active metabolites: mesoridazine, sulforidazine, and northioridazine. The use of large sample injection volumes eliminated the need for sample concentration. Thus, an extraction—back-extraction procedure was used to avoid recovery problems associated with solvent evaporation—reconstitution steps. Electrochemical detection provided excellent selectivity and sufficient sensitivity to detect less than 1 ng of phenothiazine injected onto a column. Interestingly, no northioridazine was observed in any patient sample, so that northioridazine may not be an important metabolite of thioridazine in human subjects. The present method has the capability to quantitate reliably even highly potent phenothiazines, which are typically present in blood at levels below 1 ng/ml. Compared to other HPLC methods for thioridazine and other phenothiazines, this method has several advantages in terms of improved recovery, resolution, and sensitivity.

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REFERENCES

1 B.M. Cohen, in H.C. Stancer, P.E. Garfinkel and V.M. Rakoff (Editors), The Clinical Utility of Plasma Neuroleptic Levels, Guidelines for the Use of Psychotropic Drugs, Spectrum Publications, 1983, pp. 245—260.
2 B.M. Cohen, M. Herschel and A. Aoba, Psychiatr. Res., 1 (1979) 199.
3 E.C. Dinovo, L.A. Gottschalk, B.R. Nandi and P.G. Geddes, J. Pharm. Sci., 65 (1976) 667.
4 L.A. Gottschalk, E.C. Dinovo and B.R. Nandi, Psychopharmacol. Commun., 2 (1978) 475.
5 W. Lindner, R.W. Frei and W. Santi, J. Chromatogr., 108 (1975) 299.
6 C.D. Kilts, K.S. Patrick, G.R. Breese and R.B. Mailman, J. Chromatogr., 231 (1982) 377.
7 U.R. Tjaden, J. Lankelma, H. Poppe and R.G. Muusze, J. Chromatogr., 125 (1976) 275.
8 G.L. Blackman, A.C. Ho, A. Jozsa and J.D. Kelly, J. Assoc. Anal. Chem., 63 (1980) 988.
9 S.H. Curry, E.A. Brown and J.H. Perrin, IRCS Med. Sci., 9 (1981) 168.
10 C. Bollet, P. Oliva and M. Caude, J. Chromatogr., 149 (1977) 625.
11 Technical Staff of Bioanalytical Systems, W. Lafayette, IN, 1983, personal communication.
12 J.E. Wallace, E.L. Shimek, S. Stavchansky and S.C. Harris, Anal. Chem., 53 (1981) 960.
13 K.K. Midha, J.K. Cooper and J.W. Hubbard, Psychopharmacol. Commun., 4 (1980) 107.
14 J. Fekete, P. Del Castilho and J.C. Kraak, J. Chromatogr., 204 (1981) 319.
15 LCEC Application Notes No. 41, Bioanalytical Systems, W. Lafayette, IN, 1983.