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MEASUREMENT OF BLOOD LEVELS OF NEUROLEPTICS AND METABOLITES BY COMBINED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY–RADIORECEPTOR ASSAY FOR D_2 AND SIGMA SITES

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Abstract—The dopamine (D_2) receptor blocking property of antipsychotic medications has been proposed as the mechanism of the therapeutic activity of this class of drugs. This property has also been exploited as a method to quantify therapeutic levels of these drugs in patients. However, the lack of correlation among dosage, blood levels and clinical response has resulted in a contradictory literature on both mechanism and quantification of these drugs. Bioactivity and chemical identity of the commonly prescribed neuroleptic drug fluphenazine and its metabolites in human plasma were determined by a new method which combines the selectivity of chemical methods with the sensitivity and bioassay of the radioreceptor assay (RRA) method. Fluphenazine and its metabolites were separated and identified in human plasma by an ion-pairing reverse phase high performance liquid chromatographic method with electrochemical detection. A volatile buffer system was employed which was compatible with facile sample preparation for post-column analyses, and which provided sharp, symmetrical chromatographic peaks of parent compound and metabolites. Post chromatography, HPLC fractions were assayed by RRA for D_2, alpha$_1$ and sigma receptors. More than one pattern of metabolism of the drug was seen, including biosynthesis of drug metabolites with biological activities at these receptor types. The individual differences with which this occurs may contribute to the variabilities seen in clinical response to neuroleptics, and to difficulties in neuroleptic blood level determinations.

The dopamine theory of schizophrenia is supported by the evidence that all clinically used neuroleptics are potent antagonists of ligand binding to D_2 receptors, and that the administration of neuroleptics increases dopamine levels and levels of dopamine receptors. This antagonism by neuroleptic drugs of dopamine D_2 receptors is central to the dopamine theory of schizophrenia. However, neuroleptic drugs and their metabolites have been shown to be pharmacologically active at several classes of neurotransmitter receptors, including the psychotogenic haloperidol-sensitive sigma site [1–9]. Neuroleptics have also been shown to increase levels of neurotransmitters and their receptors other than dopamine [9]. Very recently, clinically efficacious antipsychotics have been developed which do not block dopamine receptors or affect dopamine metabolism but are potent sigma receptor blockers [10]. These facts provide equally compelling arguments for neurochemical disorders of other receptor sites being the locus of some or all schizophrenic disorders. However, the most reliable point of information we have on schizophrenia is that most patients are helped by treatment with neuroleptic drugs. This suggests that the study of the pharmacological properties of these drugs may provide insights into the biochemistry of schizophrenia.

There presently exists a very poor correlation among dosage, blood levels and clinical response associated with the use of the antipsychotic neuroleptic drugs [11–14]. This lack of relationship is troubling not only in terms of classical pharmacokinetics/pharmacodynamics and hypothesized mechanisms of these drugs, but also in relation to etiologies of schizophrenia which are based upon this proposed therapeutic mechanism.

The failure to find consistent relationships among pharmacokinetic parameters of the neuroleptics may also be due to the presence of active metabolites with therapeutic or toxic effects which may be mediated by activity at other receptor sites [1, 2, 4, 15]. The presence of active metabolites of phenothiazines with therapeutic or toxic effects has been proposed as a source of error in neuroleptic blood level determinations as well as a complicating factor in understanding the actions of antipsychotic drugs [1, 2, 15]. Methodologies previously used for neuroleptic blood level determinations have not been able to differentiate parent drug and metabolites with the requisite sensitivity. The resulting pharmacokinetic data then contribute to uncertainties in understanding pharmacodynamic properties of these drugs.

At the same time there also exist serious discrepancies in blood level determinations performed using chemical methods (e.g. high performance liquid chromatography, gas chromatography) as opposed to bioassays (radioreceptor assay, radioimmunoassay). These differences are a function of the nature of the techniques employed. Chemical

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methods have had low sensitivity of detection, but may unambiguously identify molecular moieties of neuroleptics. However, metabolites with therapeutic or toxic potential may escape detection by these methods, which are directed toward physical detection of the parent compound. Radioreceptor assays (RRA*) provide a measure of biological activity in plasma, but they cannot differentiate the parent drug from active metabolites or nonspecific interference, nor are they sensitive enough for detection of high-potency, low-dose neuroleptics. The RRA for dopamine receptors of the D_2 type is performed most frequently for neuroleptic determinations, as this receptor has been proposed as the site of action of neuroleptics in the treatment of schizophrenia and related disorders [16]. However, neuroleptic metabolites with different potencies and receptor specificities may specifically affect quantification of RRA results [1, 2, 11, 13, 17, 18] and, of course, this technical strategy will not detect the biological activities of neuroleptics or their metabolites which may be mediated by other neurotransmitter receptors [4, 5, 8]. Individually, these methodologies are inadequate for the separate identification of parent compound and active metabolites and the identification of the pharmacological activities of these active metabolites.

We have developed a high performance liquid chromatographic method resolving fluphenazine (FPZ), a commonly prescribed phenothiazine, and its metabolites, with selectivity and high sensitivity provided by the use of electrochemical detection (EC). 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 other pharmacologically related compounds. Fluphenazine and its metabolites in blood samples from patients treated with FPZ are separated by HPLC, and the biological activities of parent drug and metabolites are determined by RRA of the HPLC fractions. Assays for D_2 and sigma receptor binding are performed on HPLC fractions, as actions at these receptors have been proposed as mediating the pharmacological activities of neuroleptic drugs [6–8, 10]. Data on alpha-adrenergic receptor binding assays of HPLC fractions of standard injections are also shown, to demonstrate the applicability of the method to a wide range of receptor types which may mediate other effects of these drugs.

METHODS

HPLC. A detailed ion-pairing, reverse phase HPLC method for phenothiazines has been described [19, 20], and is briefly outlined in the legend of Fig. 1. Sensitivity of the HPLC method with electrochemical detection is approximately 300 fmol [19]. For study of metabolites in blood, informed consent was obtained from inpatients prescribed oral FPZ. Blood was drawn from patients prior to the morning dose of drug, which was approximately 12 hr after the last dose. Blood (approx. 5 ml) was drawn into a vacuum tube with a plasticizer-free cap containing sodium citrate and centrifuged at 1000 *g* for 15 min. Plasma (1 ml) was pipetted into a glass screw-top test tube, 1 ml of 0.5 M K_2CO_3 was added to the tube, and the contents were vortexed. Isoamyl alcohol (10 ml) was then added, and the test tube was shaken for 10 min. Nine milliliters of the organic layer was drawn out, transferred to a second tube, and evaporated to dryness in a Speed-Vac vacuum centrifuge (Savant, Farmingdale, NY). The residue was reconstituted in 1 ml methanol or HPLC buffer, filtered through a 1-micron cellulose filter, and 10–50 μl was injected into the HPLC; the elution of phenothiazines was monitored by EC at +850 mV with a glassy carbon electrode. For chromatographic studies of fully oxidized metabolites such as sulfoxides, a dual glassy carbon electrode was employed, with the upstream electrode at a potential of −800 mV, to reduce these metabolites to an oxidizable form for detection.

HPLC fractions (2 ml) were collected each minute, lyophilized, and reconstituted for RRA. This chromatographic method provides baseline resolution of these closely related compounds with good peak shape. The buffer system is completely volatile and may be removed under vacuum prior to post-column assays. Several chromatographic runs with electrochemical detection of each patient plasma sample were routinely obtained. Subsequently, chromatographic runs were made for collection of fractions for post-column assays, during which the electrochemical cell was turned off so as not to generate electrochemically oxidized or reduced moieties during the run. Extractions of plasma itself showed no evidence of endogenous interfering substances. Recoveries of FPZ and metabolites with an isoamyl alcohol extraction were essentially complete [19].

Radioreceptor assay. Whole brains from male OM rats (for D_2 assays) or male Hartley guinea pigs (for sigma assays) were homogenized using a glass–Teflon homogenizer in 15 vol. of 50 mM Tris–HCl buffer, pH 7.4. Binding assays were run in 12 × 75 mm polypropylene tubes containing membrane suspension, tritiated ligand, unlabeled blocking ligands, lyophilized patient plasma or HPLC fraction, and buffer in a total volume of 1 ml. [^{3}H]Haloperidol (14.6 Ci/mmol; New England Nuclear, Boston, MA) was used to label D_2 sites in the presence of 10^{-6} M (+)-SKF 10,047 (*d-N*-allylnormetazocine), in a buffer of 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 [21]. Nonspecific binding was determined in the presence of 10^{-4} M (+)-butaclamol (Research Biochemicals, Natick, MA). [^{3}H]Haloperidol binding in the presence of spiperone (10^{-7} M) was used to label haloperidol-sensitive sigma sites in a 50 mM Tris–HCl buffer, pH 7.4 [6–8]. Nonspecific binding was determined with 1 μM (+)-SKF 10,047. Alpha$_1$ sites were assayed with [^{3}H]prazosin, with specific binding determined with 100 μM phenoxybenzamine HCl, in a 50 mM Tris–HCl buffer, pH 7.7 [22]. An aliquot of each patient's plasma, extracted as described above, was assayed without HPLC for total binding activity along with the fractions comprising the entire chromatographic run. Recovery of binding activity after HPLC was essentially quantitative, as individual

* Abbreviations: RRA, radioreceptor assay; EC, electrochemical detection; and FPZ, fluphenazine.

metabolites could be chromatographed, dried under vacuum, and re-chromatographed without significant loss (not shown).

After a 45-min incubation at 25°, the contents of each tube was rapidly filtered through Whatman GF/B glass-fiber filters, which were presoaked in 0.1% polyethylenimine, followed by washing with ice-cold buffer (2 × 10 ml), in a cell harvester apparatus (Brandel, Gaithersburg, MD). Filtration was completed within 15 sec. The filters were counted in 10 ml Readisolv after vigorous shaking. Sensitivities of all the binding assays were found to be in the low nanomolar range.

RESULTS

Chromatography of standard injections of FPZ and metabolites (600 fmol each) are shown (Fig. 1A), followed by RRA of chromatographic runs of the same samples for D_2, alpha$_1$, and sigma receptor sites (Fig. 1, B–D respectively). Fluphenazine and metabolites demonstrated baseline resolution with sharp and relatively symmetrical peaks, in a single short chromatographic run. Radioreceptor assays of these same chromatographic runs provided bioactivity data for all these compounds at D_2 (Fig. 1B), alpha$_1$ (Fig. 1C) and sigma (Fig. 1D) binding sites.

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. Both patients were administered the same dosage (5 mg/day, b.i.d., p.o.) for approximately 5 days. Plasma from patient A appeared to contain FPZ, 8- and 7-hydroxyl-FPZ, and the *N*-oxide metabolite (Fig. 2A). Radioreceptor assays of chromatographic fractions of this patient's plasma (Fig. 2, B and C) demonstrated activity at D_2 receptors of the parent compound as well as of the *N*-oxide, 7-hydroxyl, and to a lesser extent, the sulfoxide. The sigma receptor assay of this HPLC run indicated the high level of sigma activity of FPZ and of several of the metabolites.

In contrast, patient B (Fig. 3A) had a much higher plasma level of FPZ and did not appear to metabolize it in the same manner as patient A. Radioreceptor assays of chromatographic fractions of the plasma of

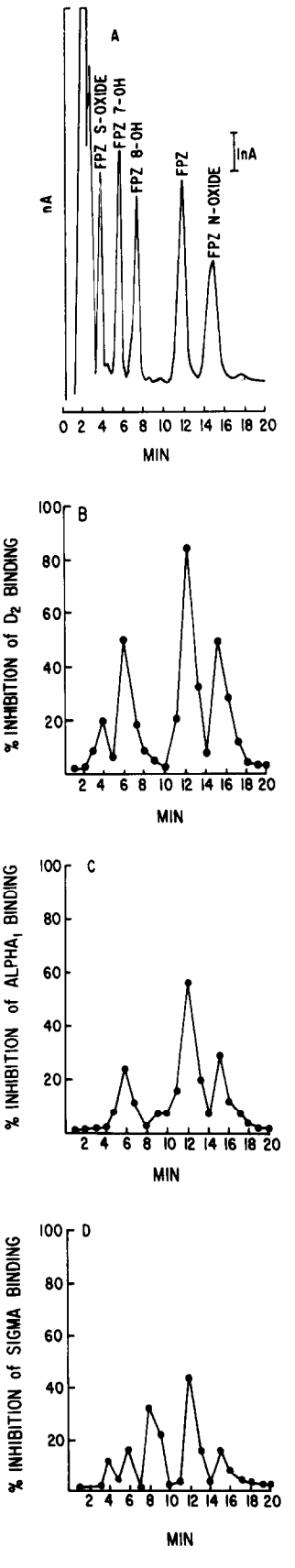

Fig. 1. Representative chromatogram of a standard injection of FPZ and metabolites extracted from spiked plasma, with subsequent RRA. The mobile phase was 0.15 M formic acid and 0.01 M dibutylamine in 50% methanol, pH 3.2. The flow rate was 2 ml/min, and the column was maintained at 40°. The column effluent was first passed over a glassy carbon electrode set at a potential of −800 mV, to reduce oxidized metabolites. Detection took place at a second glassy carbon electrode in series set at a potential of +850 mV versus a Ag/AgCl reference electrode. Fractions were collected for post-column assays from chromatographic runs with the electrodes at 0 V potentials, to avoid electrochemical reactions in the chromatographic system. (A) HPLC–EC of standards. (B–D) Radioreceptor assays of the chromatographic runs of the same samples: (B) D_2; (C) alpha$_1$; (D) sigma. Inhibition of binding by 50% is approximately equal to a concentration of competing ligand of 100–200 nM.

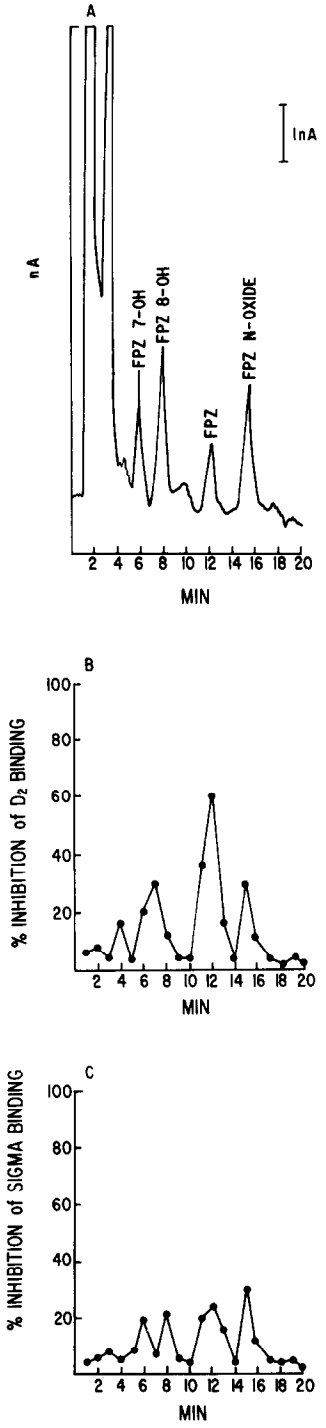

Fig. 2. HPLC–RRA applied to plasma extract of patient plasma. Chromatography as in Fig. 1. (A) HPLC–EC of patient A plasma. (B and C) Radioreceptor assays of these chromatographic runs: (B) D_2; (C) sigma.

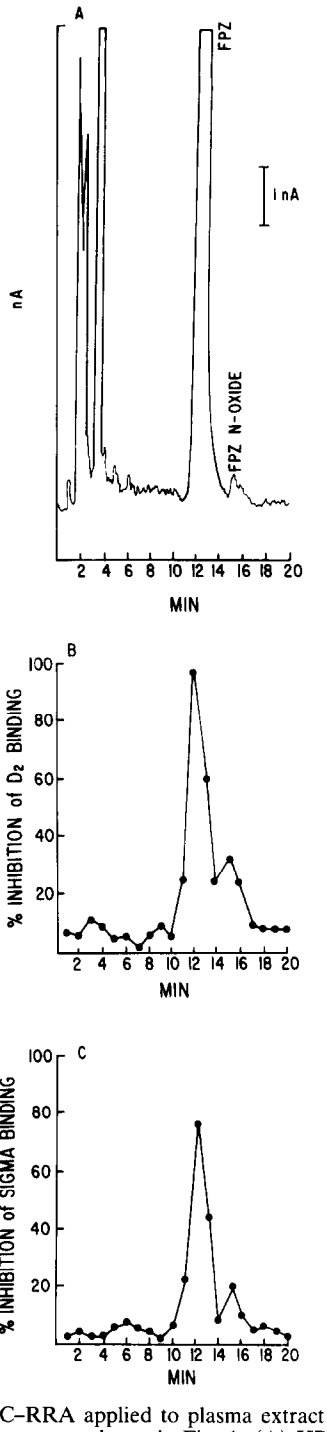

Fig. 3. HPLC–RRA applied to plasma extract of patient plasma. Chromatography as in Fig. 1. (A) HPLC–EC of patient B plasma. (B and C) Radioreceptor assays of these chromatographic runs: (B) D_2; (C) sigma.

patient B (Fig. 3, B and C) reflected this pattern, with all the D_2 activity in the FPZ peak, except for a minor *N*-oxide component, which could not be seen in the HPLC trace. Sigma activity assay of this patient's plasma demonstrated activity attributable to FPZ and, at a much lower level, to FPZ *N*-oxide. Clinically, patient A was considered to have a more satisfactory response to medication, while patient B demonstrated more sedation on this dose of FPZ.

DISCUSSION

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 [19, 20, 23–25]. Methodological limitations inherent in studies of antipsychotic drug pharmacokinetics were mitigated by the combination of HPLC with RRA, which provided all the advantages of each of these techniques alone, as well as other advantages unique to the combined method.

Both the 7-hydroxyl and *N*-oxide metabolites of FPZ demonstrated binding to D_2 and alpha$_1$ sites, while the sulfoxide metabolite had little activity (Fig. 1), as has been noted in part previously [4]. Of particular interest was the apparent activity of these metabolites and the parent compound at the haloperidol-sensitive sigma site, which may mediate the psychotomimetic effects of certain opiates, and possibly the antipsychotic activity of neuroleptic drugs. These metabolites, and several others, were seen in the plasma of some (Fig. 2) but not all (Fig. 3) patients taking FPZ. In six other patients in which data are not yet complete and whose data are not presented in this paper, both the 7-hydroxyl and *N*-oxide metabolites were found in plasma. In two out of these six patients the 8-hydroxyl metabolite was also seen. These data may be of clinical significance regarding the contribution of active metabolites to both the therapeutic and adverse effects of neuroleptics.

The use of haloperidol as a ligand in both the D_2 and sigma assays provides both advantages and disadvantages. Haloperidol is relatively unstable for use as a radioactively labeled ligand, and its chemical decomposition must be monitored carefully. It also has a high nonspecific binding which requires the appropriate blocking agents. On the other hand, it has been used successfully in radioligand binding assays in the past [3, 7, 8] and as a commonly-prescribed antipsychotic drug provides a clinically relevant assay of antipsychotic drug levels [7]. Other ligands for this site may be used, as may ligands for other binding sites of interest, e.g. cholinergic and opioid.

Pharmacokinetic parameters such as metabolism may be central to the understanding of idiosyncratic responses to psychoactive drugs. These individual variations can affect significantly patient response to the clinical use of the neuroleptic drugs, including the phenothiazines. Drug metabolism can also make quantification of neuroleptic blood levels difficult, by the production of interfering metabolites. Metabolism may change the total bioavailability of antipsychotic medicine and also influence the occurrence of adverse effects of the medication. Such adverse effects may be mediated by drug metabolites active at other receptor sites, such as cholinergic or alpha-adrenergic receptors [1–4, 9, 11, 17].

Post-column analyses of the HPLC fractions such as RRA can provide greater sensitivity than most physical methods for the detection of neuroleptics, as well as specific information on the bioactivity of the parent drug and metabolites. The RRA may be utilized to enhance the sensitivity and selectivity of detection of the HPLC method. Quantification by HPLC–EC may be verified by the RRA techniques. In addition, sampling techniques, drugs or endogenous substances other than the compound of interest, including components of plasma and serum, may interfere with the quantification of neuroleptic levels by RRA alone [11, 13, 17]. Prior HPLC can serve to eliminate interference in the RRA by metabolites, other drugs, endogenous substances, serum or plasma, and so increase the sensitivity of the post-column assays by improving the signal:noise ratio. This method may also serve as a rapid screen for the biosynthesis of biologically active metabolites of antipsychotic drugs.

This procedure, which represents a novel combination of these two methods, offers the chemical specificity of HPLC with the assay of pharmacological activity and sensitivity of RRA. Such chemical identity is particularly important in view of the possible role of active metabolites of neuroleptics, especially FPZ, in both the therapeutic and toxic effects of these drugs [1, 2, 4, 15]. This combination offers versatility as well as a means of intra-assay verification of peak identity and quantification. Prior HPLC with the volatile buffer system employed also eliminates nonspecific interference from plasma or other drugs in subsequent bioassays such as RRA. We have the opportunity with this method to identify levels of parent drug and active metabolites at some of their putative sites of biological activity.

It is now clear that neuroleptic drugs and their metabolites are pharmacologically active at several classes of neurotransmitter receptors [1–10, 15]. The activities of neuroleptics and their metabolites at multiple receptor sites may play roles in both the therapeutic and toxic actions of these drugs. Idiosyncrasy in metabolism may be a major factor in the variability in pharmacokinetic data which appears in the literature. Taking into account the presence of metabolites of neuroleptics with therapeutic or toxic effects may finally permit the establishment of consistent relationships among pharmacokinetic parameters and clinical efficacies of the neuroleptics. The method provides both basic and clinical data, offering improved patient care and data supporting or questioning specific neurotransmitter receptor theories of schizophrenia and antipsychotic drug action.

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REFERENCES

1. Dahl SG, Active metabolites of neuroleptic drugs: Possible contribution to therapeutic and toxic effects. *Ther Drug Monit* **4**: 33–40, 1982.
2. Dahl SG, Hough E and Hals P-A, Phenothiazine drugs and metabolites: Molecular conformation and dopaminergic, adrenergic and muscarinic cholinergic receptor binding. *Biochem Pharmacol* **35**: 1263–1269, 1986.
3. Fulton A and Burrows GD, ^{3}H-Haloperidol binding to tissue receptors: Influence of some drugs and antipsychotic potency. *Life Sci* **26**: 1505–1508, 1980.
4. Hals P-A, Hall H and Dahl SG, Phenothiazine drug metabolites: Dopamine D2 receptor, alpha$_1$- and alpha$_2$-adenoreceptor binding. *Eur J Pharmacol* **125**: 373–381, 1986.

5. Richelson E and Nelson A, Antagonism by neuroleptics of neurotransmitter receptors of normal human brain *in vitro*. *Eur J Pharmacol* **103**: 197–204, 1984.
6. Su T-P, Evidence for *sigma* opioid receptor: Binding of [^{3}H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. *J Pharmacol Exp Ther* **223**: 284–290, 1982.
7. Su T-P, Schell SE, Ford-Rice FY and London ED, Correlation of inhibitory potencies of putative antagonists for sigma receptors in brain and spleen. *Eur J Pharmacol* **148**: 467–470, 1988.
8. Tam SW and Cook L, Sigma opiates and certain antipsychotic drugs mutally inhibit (+)-[^{3}H]SKF 10,047 and [^{3}H]haloperidol binding in guinea pig brain membranes. *Proc Natl Acad Sci USA* **81**: 5618–5621, 1984.
9. Cohen BM and Lipinski JF, *In vivo* potencies of antipsychotic drugs in blocking alpha$_1$ noradrenergic and dopamine D_2 receptors: Implications for drug mechanisms of action. *Life Sci* **39**: 2571–2580, 1986.
10. Ferris RM, Tang FL, Chang KJ and Russell A, Evidence that the potential antipsychotic agent rimcazole (BW 234U) is a specific, competitive antagonist of sigma sites in brain. *Life Sci* **38**: 2329–2337, 1986.
11. Ko GN, Korpi ER and Linnoila M, On the clinical relevance and methods of quantification of plasma concentrations of neuroleptics. *J Clin Psychopharmacol* **5**: 253–262, 1985.
12. Rivera-Calimlim L and Hershey L, Neuroleptic concentrations and clinical response. *Annu Rev Pharmacol Toxicol* **24**: 361–386, 1984.
13. Cooper TB, Plasma level monitoring of antipsychotic drugs. *Clin Pharmacokinet* **3**: 14–28, 1978.
14. Harris PQ, Friedman MJ, Cohen BM and Cooper TB, Fluphenazine blood levels and clinical response. *Biol Psychiatry* **17**: 1123–1130, 1982.
15. Lewis MH, Widerlov E, Knight DL, Kilts CD and Mailman RB, *N*-Oxides of phenothiazine antipsychotics: Effects on *in vivo* and *in vitro* estimates of dopaminergic function. *J Pharmacol Exp Ther* **225**: 539–545, 1983.
16. Burt DR, Creese I and Snyder SH, Antischizophrenic drugs: Chronic treatment elevates dopamine receptor binding in brain. *Science* **196**: 326–328, 1977.
17. Suckow RF, Problems in sampling techniques for psychotropic drug assays. *J Liquid Chromatogr* **10**: 293–304, 1987.
18. Tune LE and Coyle JT, Neuroleptic drug level monitoring in psychiatry: Focus on radioreceptor assay techniques. *Ther Drug Monit* **4**: 59–64, 1982.
19. Hoffman DW, Edkins RD, Shillcutt SD and Salama A, New high-performance liquid chromatographic method for fluphenazine and metabolites in human plasma. *J Chromatogr* **414**: 504–509, 1987.
20. Hoffman DW, Edkins RD and Shillcutt SD, Human metabolism of phenothiazines to sulfoxides determined by a new high performance liquid chromatography-electrochemical detection method. *Biochem Pharmacol* **37**: 1773–1777, 1988.
21. Seeman P, Ulpian C, Wreggett KA and Wells JW, Dopamine receptor parameters detected by [^{3}H]spiperone depend on tissue concentration; Analysis and examples. *J Neurochem* **43**: 221–235, 1984.
22. Greengrass P and Bremner R, Binding characteristics of ^{3}H-prazosin to rat brain alpha-adrenergic receptors. *Eur J Pharmacol* **55**: 323–326, 1979.
23. Breyer U, Gaertner HJ and Prox A, Formation of identical metabolites from piperazine- and dimethylamino-substituted phenothiazine drugs in man, rat and dog. *Biochem Pharmacol* **23**: 313–322, 1974.
24. Breyer U, Prox A, Bertele R and Gaertner HJ, Tissue metabolites of trifluoperazine, fluphenazine, prochlorperazine and perphenazine in the rat: Identification and synthesis. *J Pharm Sci* **63**: 1842–1848, 1974.
25. Goldstein SA and Van Vunakis H, Determination of fluphenazine, related phenothiazine drugs and metabolites by combined high-performance liquid chromatography and radioimmunoassay. *J Pharmacol Exp Ther* **217**: 36–43, 1981.