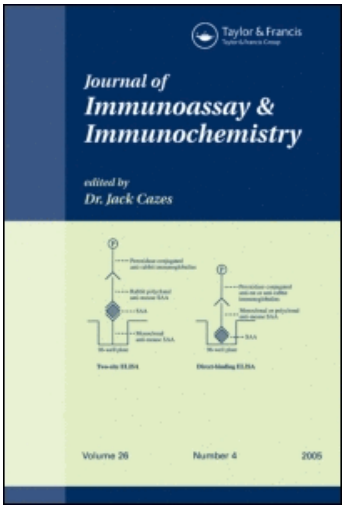

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Quantification of Reduced Haloperidol and Haloperidol by Radioimmunoassay

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QUANTIFICATION OF REDUCED HALOPERIDOL AND HALOPERIDOL BY RADIOIMMUNOASSAY

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ABSTRACT

A radioimmunoassay for reduced haloperidol and haloperidol has been developed by using a simple derivatization-separation step prior to assay with an antibody cospecific for both compounds. The detection limit of the assay is less than 25 pg and shows no cross reactivity to other metabolites. The intraassay coefficient of variation for reduced haloperidol and haloperidol were 9.0 and 8.2% respectively and the interassay coefficients of variation were 9.0 and 10.6% respectively at 5-10 ng/ml. As many as 30 patient samples can be analyzed for both compounds in a single day.

(KEY WORDS: Radioimmunoassays. Drug Analysis. Drug Metabolism. Metabolites. Binding Assays. Immunochemical Methods).

INTRODUCTION

Haloperidol (H) is one of the most commonly prescribed agents for the treatment of schizophrenia. Like most other antipsychotic agents, H is a dopamine

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receptor antagonist. Some studies (1,2,3) have shown a large inter-individual variation in blood levels among patients on the same dosage. Although Forsman et al. (4) have concluded there is "no simple relationship between pharmacokinetics of H and the clinical response", others show a correlation between blood level and therapeutic response (5,6).

When radioreceptor assays are utilized to measure H blood levels there is often a poor correlation with other analytical techniques (7,8). However, radioreceptor assay of H has been shown to correlate well with radioimmunoassay and gas chromatography-mass spectrometry measurement when blood levels are measured after an acute dosage (9). This difference is generally ascribed to the presence of metabolites in the chronically treated patient (10).

In this respect H has a unique metabolic profile. Oxidative N-dealkylation yields two smaller fragments which have no pharmacological activity (11, 12). However, H can form a reduction product in which the benzylic ketone is reduced to an alcohol (13). This metabolite, (reduced haloperidol) designated RH, is the only metabolite of H known to possess neuroleptic activity (4). We found that RH is equipotent to H in displacement of ^{3}H-H in studies to determine

the crossreactivity of RH with an antibody developed in our laboratory to H. This finding was utilized to develop an analytical technique to measure RH. Recently two groups have reported liquid chromatographic methods for determining H and RH (14,15). One method (15) utilizes an absorbance detection method and as such does not have requisite sensitivity. The other method (14), utilizing electrochemical detection is sensitive enough but is still cumbersome and time consuming. Several radio immunoassays (RIA) have been developed for H (16,17,18). However, antibodies against haloperidol linked at either end of the H molecule overestimate patient plasma levels by 40%-200%, as compared to gas-chromatography (GC), when no extraction is utilized prior to RIA (19,20).

Thus, the purpose of this study was to develop a reliable and rapid means of quantifying RH and H in patient and animal samples. Additionally, we examined the relationship between blood levels of H and RH in a number of samples from patients undergoing chronic haloperidol therapy.

MATERIALS AND METHODS

Haloperidol and its metabolites were a gift of McNeil Laboratories, ^{3}H-H was obtained from Research

Products International (RPI) and checked for purity before use. All other chemicals used were reagent grade and purchased from common chemical sources.

Preparation of Immunogen

Haloperidol-oxime was prepared by reaction of H with carboxymethoxylamine by a modification of the method of Erlanger, et al (21). Briefly, 0.9 mmole carboxymethoxylamine was reacted with 0.5 mmole H in ethanol made acidic by addition of acetic acid. The reaction product was subsequently extracted with chloroform and dried in vacuo. Haloperidol-oxime was then conjugated to charcoal cleaned BSA (22), by the mixed anhydride method of Vaughn (23). The conjugate, contained a molar ratio H/BSA of 26/1 as determined by addition of ^{3}H-H.

Immunization Procedures

The conjugate, containing a molar ratio H/BSA of 26/1, was suspended in 0.9% NaCl and mixed with equal volumes of Freund's Complete Adjuvant to give a final concentration of 5 mg/ml.

White New Zealand rabbits housed in our animal care facilities were given 0.5 ml intramuscular injections in each flank. Booster injections were given monthly for six months and arterial blood was drawn from the ear seven days after the last injection.

Radioimmunoassay

The 0.05 M Borate buffer (pH 8.0) used with all assays and dilutions contained 1 mg/ml of gelatin. The RIA contained the following: 0.5 ml antibody diluted 1:17,000 (final dilution 1:20,000), 152 pg ^{3}H-H in 50 µl (16,000 dpm, 19 Ci/mmole) and 50 µl of sample or standard in .05N HCl. After the assay was incubated 1 hr. at room temperature, the antibody bound H was separated from free H by addition of 200 µl dextran coated charcoal (3% Norite A charcoal coated with 0.3% Dextran T70). After 10 minutes the charcoal containing assay tubes were centrifuged at 1,000 xg for 2 minutes. A 0.5 ml aliquot of the supernatant was withdrawn and assayed for radioactivity by scintillation spectrometry using a Nuclear Chicago Mark II liquid scintillation spectrometer.

Total immunoactivity was measured directly after extraction while H was determined after extraction and specific removal of RH by derivatization and chromatography. RH was taken to be the difference between total immunoactivity (TI) and H values (e.g., TI -H = RH). Sample or standard (0.5 ml) in plasma with 6,000 dpm ^{3}H-H added for recovery was extracted by addition of 20 µl isoamyl alcohol, 4.5 ml pentane, and 200 µl saturated sodium carbonate. The extraction

mixture was shaken for 1 hr. and then centrifuged 10 min. at 2,000 xg. The lower aqueous layer was frozen at -80°C and the upper organic phase (containing the H and RH) was transferred into two other tubes each containing approximately one half of the extract (one tube for TI and one for H, determination) and dried under N_2. In order to determine H specifically, RH must be derivitized prior to silica gel chromatography, since underivatized RH was not separable by chromatography alone. Thus, 100 µl of a derivatization mixture was added to one half of the dried extract. This mixture was prepared just prior to its use by saturating a 5% trimethylamine in benzene solution with an excess of succinic anhydride. The 5% (by weight) solution was prepared by bubbling trimethylamine into redistilled benzene, and may be stored at 4°C in the dark for up to 6 weeks without deterioration. The derivatization was carried out at room temperature for 1 hr.

Derivatized product was separated from underivatized product on silica gel columns which were prepared by addition of about 150 mg silica gel (60-200 mesh) to a small (10 ml) polypropylene column (Biorad, Richmond, CA). These columns were then washed with 1 ml pentane, 1 ml ethanol, then 1 ml elution solvent which consisted of pentane/ethanol/NH_4OH (50:50:0.25). The extracted, derivatized sample was diluted with 1 ml of the elution solvent and transferred to the column. The sample tubes

were rinsed again with 1.0 ml elution solvent which was also applied to the column. The combined elution solvent containing H was dried under N_2 at 40°C. Both the extracted, derivatized, and chromatographed sample (H) and the extracted only sample (TI) were reconstituted to 0.5 ml with 0.05N HCl. An aliquot (200 µl) was assayed for radioactivity to determine recovery from the underivatized and derivatized extracts and 50 µl in duplicate were assayed for H or TI by RIA. Using these procedures, as many as 30 samples may be analyzed for both H and RH in a single day.

Liquid Chromatography Assay

Samples analyzed by liquid chromatography were processed by a slight modification of that described by Korpi, et al., (14). Briefly, 2 ml plasma samples containing 10,000 dpm ^{3}H-H for recovery were alkalinized and extracted twice for 15 minutes each time with 5 ml of the extraction solvent (1.5% isoamyl alcohol in heptane). The samples in the organic phase were then extracted into .2N HCl and the acid layer washed with extraction solvent. Finally the aqueous layer was alkalinized and extracted twice with extraction solvent which was then dried under N_2 and reconstituted in 250 µl mobile phase. The mobile phase consisted of potassium phosphate buffer 80 mM (adjusted to pH 6.8) and glass redistilled HPLC-grade

acetonitrile, 50/50 (by vol). One fifth of each sample (50 μl) was injected onto a Custom LC, 5 micron cyanopropylsilane column (.46 x 25 cm) using a Valco injection valve. Samples were analyzed with a Bioanalytical LC 2A controller operated at +.9V potential with a glassy carbon TL5 flow cell. Concentrations of RH and H were determined by peak height comparison with a standard curve and corrected for recovery by liquid scintillation analysis of ^{3}H-H present in the extract. Recovery was typically greater than 95%.

RESULTS AND DISCUSSION

Radioligand:

Figure 1 shows radiochromatograms of ^{3}H-H from two different sources that were analyzed by High-Performance Thin-Layer Chromatography (HPTLC) using $CHCl_3$/ ethyl acetate (4:1) as a solvent system in a chromatography chamber saturated with NH_4 vapor. The two lots from Research Products International (RPI) (Fig. 1A, B) and one lot from New England Nuclear, (NEN) (Fig. 1C) reveal significantly different levels of purity from lot to lot and vendor to vendor. All three labeled products, as well as authentic standards were chromatographed simultaneously. Ultraviolet and radioactive densitometric scans revealed the major radioactive peak in all three

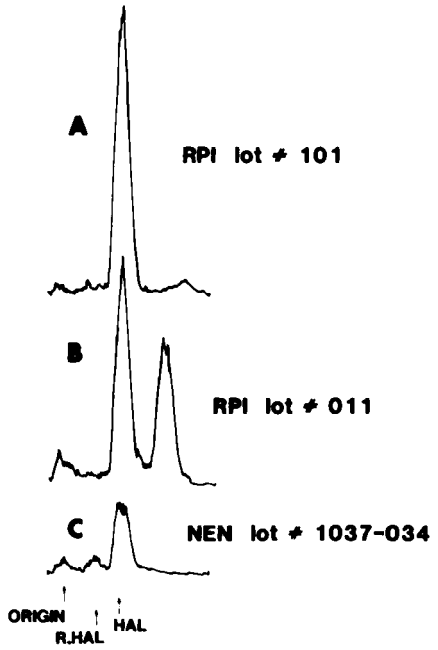


Figure 1. HPTLC radiochromatograms of ^{3}H haloperidol from two sources: Research Products International (RPI) and New England Nuclear (NEN). Arrows mark R_f of authentic standards. Details in text.

preparations co-chromatographed with authentic H. (R_f = 0.39). The minor peak in the RPI preparations appears to contain a ketone function that is not benzylic (reacts slowly with carboxymethoxylamine). The minor peak in the New England Nuclear product co-chromatographed with RH (R_f = 0.20). Our experience with RIA and radioreceptor assay indicate that homogeneity of radioligand may be an

important issue in the quality of results obtained by competitive protein-binding assays. These results indicate that since each lot of ^{3}H-H can vary substantially in purity, they should be checked routinely.

Standard Curve:

Anti-H-BSA serum was used at a dilution of 1:20,000 using 152 pg (16,000 dpm) ^{3}H-H from Research Products International. The standard curve for H was linear on a Logit B/B_0 as shown in Figure 2. The inset shows a Eadie-Hofstee analysis of binding revealing a Ka of 4.02×10^{9}M. The assay was sensitive to less than 25 pg (0.5 ng/ml) using two standard deviations from the mean as the limit.

Cross reactivity:

The assay was tested for interference by H metabolites and related compounds (Figure 3). RH was equipotent in displacing ^{3}H-H. This co-specificity is due to the hapten linkage to BSA occuring at the site of reduction of the H molecule. The subtraction H from TI to obtain RH concentration utilizing one antibody is to our knowledge unique but could be utilized in other assays where two compounds are of very similar structure. Poland and Rubin (24) have utilized a similar approach to measure H and RH. Their technique utilized two different antibodies

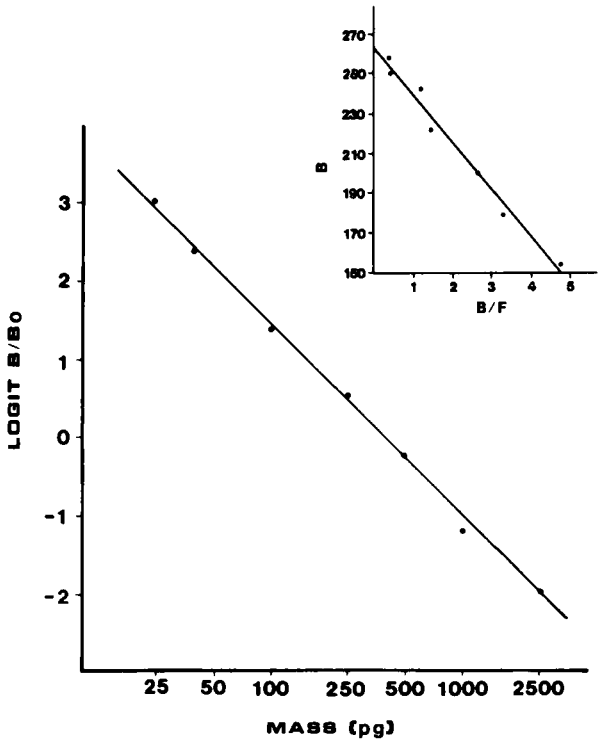


Figure 2. Standard curve using haloperidol. Practical sensitivity of assay using a 50 µl plasma aliquot and sensitivity of 25 pg was 0.5 ng/ml. The inset shows an Eadie-Hofstee analysis of the data plotted as Bound/Free versus Bound.

directed toward different ends of the molecule. The two antibody approach could be effectively employed only if one antibody was absolutely specific for one compound while the other only detected both compounds. However, Rubin et al have shown that an

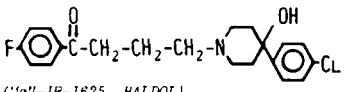
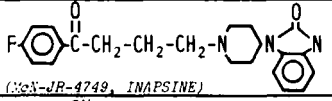
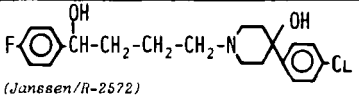
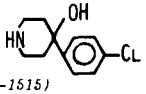
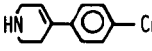
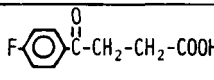
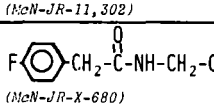
COMPOUND NUMBER	STRUCTURE AND REFERENCE NUMBER	GENERIC NAME	AMOUNT WHICH REDUCES ANTIBODY BINDING OF ^{3}H-HALOPERIDOL TO 50%
1	 (McN-JR-1625, HALDOL)	HALOPERIDOL	1
2	 (McN-JR-4749, INAPSINE)	DROPERIDOL	2.5×10^4
3	 (Janssen/R-2572)	REDUCED HALOPERIDOL	1
4	 (McN-JR-1515)	SUBSTITUTED PIPERIDINYL MOIETY OF HALOPERIDOL	3.1×10
5	 (McN-JR-2576)	DEHYDRATION PRODUCT OF COMPOUND 4	7.2×10^2
6	 (McN-JR-11,302)	HALOPERIDOL METABOLITE	3×10^8
7	 (McN-JR-X-680)	HALOPERIDOL METABOLITE	1.4×10^5

Figure 3. Crossreactivity of haloperidol metabolites and butyrophenones with oxymo-haloperidol specific antiserum.

antibody produced against succinyl-H, where the hapten is linked through the tertiary alcohol function at the other end of the molecule by means of reaction with chlorosuccinic acid, cross reacts with other H metabolites although not RH (19). Also this technique would require two different derivatizable

moieties on opposite sides of the molecule. While While this might be possible for H, it would not be for many other compounds. The only other known metabolite which competed with our antibody significantly was the piperidinyl moiety (Figure 3), which required 31-fold higher concentrations to produce a comparable displacement when added directly to the immunoassay. However, the piperidinyl moiety did not extract and was undetectable at 1 μg (20 μg/ml) in the complete assay.

Assay:

Recoveries were routinely accounted for by addition of a small amount (6,000 dpm) of ^{3}H-H. Of 16,000 dpm added to the RIA incubation only 420 dpm in the final assay aliquot could be attributed to the recovery addition. Recovery of H from the columns was >98%, although recovery through extraction, derivatization and chromatography was generally about 85%. Addition of a ^{3}H-H tracer increases precision by monitoring the recovery of the extraction derivatization, and chromatography steps. Tritiated RH prepared by reduction of ^{3}H-H showed that H and RH extract equivalently in this assay. No loss of H due to derivatization by succinic anhydride was detectable under the conditions employed above. Even when incubated at 60°C for one week a loss was noted only

when an extremely high molar ratio of succinic anhydride to H was used (10^6:1). Additionally no RH was detected in the H assay indicating derivatization and chromatography allowed complete removal of RH. Formation of a succinyl derivitive in order to facilitate chromatographic separation in a RIA is to our knowledge a novel idea. However, succinylation and acetylation procedures have been utilized in RIA's in order to improve immunologic specificity and sensitivity (25; 26) (Table I). The intraassay variation was 8.2% for H (n=10) and 9.0% for RH determinations (n=12) (Table I). Interassay variation was 10.6% for H determinations (n=11) and 9.0% for RH (n=11). Quality control standards containing equal amounts of H and RH were routinely included in all assays. Using this procedure over 1000 samples have been determined in our laboratory during the last 3 years.

Clinical:

Figure 4 shows the correlation between RH plasma levels and H plasma levels in 100 patients chronically treated with H. This figure suggests that as plasma levels of H are increased above about 20 ng/ml the RH levels are increased at a much more rapid rate than H. Above 20 ng/ml the body appears to metabolize H to RH in increasingly greater amounts. The significance of this metabolism in determining the thera-

TABLE 1 ***Assay Precision and Accuracy***

MASS ADDED	MASS MEASURED	
	HALOPERIDOL (NG/ML)	HALOPERIDOL + REDUCED HALOPERIDOL (NG/ML)
6.25 NG/ML HALOPERIDOL	5.84 ± 0.48 (10)	6.40 ± 0.34 (10)
25.00 NG/ML HALOPERIDOL	25.53 ± 1.80 (10)	25.18 ± 1.23 (10)
10.00 NG/ML REDUCED HALOPERIDOL	0.58 ± 0.79 (3)	9.76 ± 0.88 (12)
10.00 NG/ML HALOPERIDOL + 10.00 NG/ML REDUCED HALOPERIDOL	8.92 ± 0.40 (3)	19.63 ± 0.50 (3)

peutic effect of H needs to be explored in a controlled clinical situation.

Comparison:

Six of these patient samples which had a wide range of concentrations of RH (8.4-56.6 ng/ml) and RH to H ratios (.34-2.8) were subjected to analysis by both RIA and Liquid Chromatography. Table II shows the comparison of values determined by HPLC-electrochemical detection technique (14) and RIA. Both analytical methods yield values which are the same within 95% confidence as judged by a paired t-test. These results suggest that only RH and H are detected in this assay. Although HPLC and RIA yield

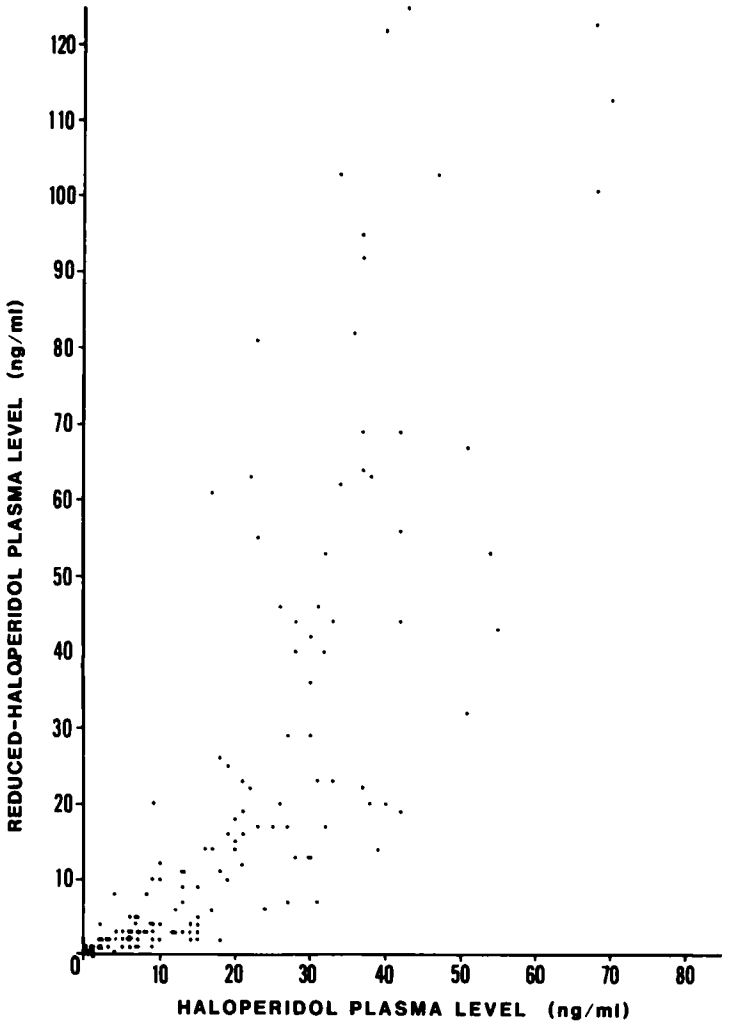


Figure 4. Correlation of haloperidol plasma level (ng/ml) with reduced haloperidol plasma level (ng/ml) in 100 patients chronically treated with haloperidol.

TABLE 2

COMPARISON OF LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY VALUES

	HALOPERIDOL ng/ml		REDUCED HALOPERIDOL ng/ml	
	LC	RIA	LC	RIA
1.	24.2	25.3	9.5	11.6
2.	20.7	24.5	8.4	8.4
3.	14.5	12.9	7.5	12.2
4.	42.8	40.9	52.2	56.6
5.	17.7	11.4	25.3	32.3
6.	13.4	11.8	33.3	29.2

similar results, there are several advantages in the RIA procedure. HPLC requires a much larger sample volume (2-4 ml) compared to RIA (0.5 ml). The entire sample must be applied to the HPLC column in order to achieve comparable sensitivity to RIA. Since only one tenth of the sample is assayed in the RIA, analysis of a larger aliquot (50% or greater) would increase sensitivity if desired. In addition, the time required to analyze a set of samples by HPLC is two to three times that of the RIA. Although sample preparation times are equivalent (three hours), analysis by RIA

requires less than 1/3 of the time required of HPLC. Due to late eluting peaks seen in some samples HPLC may require even more time than is assumed above. Finally, electrochemical HPLC requires a highly skilled technician using instrumentation that is available to few clinical laboratories. Thus we feel that our RIA is a more sensitive, simple, inexpensive and rapid analytical approach than any others currently available.

Conclusion:

The development of an assay procedure which quantitates the absolute amount of all active drug components (parent and metabolites) provides the best opportunities to evaluate the relationship between drug levels in plasma and therapeutic effect. Haloperidol is a unique neuroleptic in that it has been reported to have only one active metabolite (RH) and as such appears to offer this study opportunity. A similar study involving one of the phenothiazines would require individual measurements of many active metabolites (27) and would be much more difficult to carry out and to evaluate the contribution of each component to the therapeutic effect.

Our preliminary evidence indicates a lack of therapeutic efficacy of H in patients exhibiting extremely high RH to H ratios (28).This may imply that RH plays a role in determining the therapeutic

efficacy of H. This high ratio may be due to the longer half-life of RH when compared to H (28) or variable conversion of H to RH in the gut (Browning, Harrington and Davis, submitted for publication). In summary, the radioimmunoassay technique reported here, provides a unique, rapid and reliable method for quantitation of H and RH in plasma. This work further suggests the possible utility of measuring the concentration of RH relative to H as a potential factor in predicting therapeutic efficacy.

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ABBREVIATIONS

BSA - bovine serum albumin

RIA - radioimmunoassay

HPLC - high pressure liquid chromatography

Ka - association affinity constant

H - haloperidol

RH - reduced haloperidol

TI - total immunoactivity

GC - gas chromatography

LC - liquid chromatography

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