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Sensitive method for the assay of sertindole in plasma by high-performance liquid chromatography and fluorimetric detection

Tsang-Bin Tzeng*, Gaynelle Stamm, Sou-yie Chu

Drug Metabolism Department, Abbott Laboratories, One Abbott Park Road, Abbott Park, IL 60064-3500, USA

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Abstract

A simple and highly sensitive normal-phase HPLC method is described for determining sertindole concentrations in human plasma using fluorimetric detection. A short C_8 column was used to extract sertindole and the internal standard from plasma; the column was rinsed with acetonitrile, and the analytes were recovered by elution with methanol. This uncommon selectivity between the two solvents allowed clean extraction and near- quantitative recovery of the analytes ($>89\%$). Separation was done on a 5-μm silica-gel column and detection was performed by fluorimetry, with emission at 340 nm and excitation at 260 nm. The detection and lower quantifiable limits were 0.01 and 0.025 ng/ml, respectively, with no interference from plasma or potential metabolites.

1. Introduction

Sertindole is a new imidazolidinone serotoninergic and dopaminergic antagonist with selective effects on mesolimbic but not on nigrostriatal dopaminergic neurons (Fig. 1). Because of this selectivity, the drug may possess neuroleptic properties with minimal or no neurological side effects when given in clinical regimens [1–5]. Sertindole is currently being developed as a potential antipsychotic agent. The major challenge in developing a method to quantitate sertindole in serum or plasma is achieving adequate sensitivity. The high in vitro potency of the compound and the anticipated low clinical dosages require a lower limit of quantitation of 0.05 ng/ml, or possibly even lower. Initially a normal-phase high-performance liquid chromato-

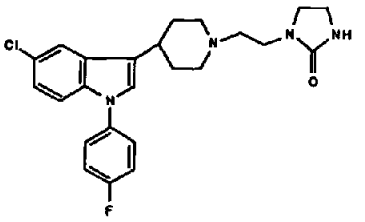

Fig. 1. Structure of sertindole.

* Corresponding author. Present address: Department of Pharmaceutical Sciences, School of Pharmacy, Temple University, 3307 N. Broad Street, 602-00 Philadelphia, PA 19140, USA.

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graphic (HPLC) method with UV detection was developed that provided a sensitivity down to 0.5 ng/ml using 2-ml samples. It was noticed that sertindole had very little fluorescent properties in aqueous media. However, scientists at Shionogi Pharmaceutical Co., Japan, found that in organic media, assay sensitivity for sertindole can be improved by using fluorimetric detection [6]. We have combined this mode of detection with a novel and simple solid-phase extraction technique to provide a rapid, specific, sensitive, and robust normal-phase HPLC method for the assay of sertindole in human plasma or serum. The method has been used successfully in Phase I pharmacokinetic studies here at Abbott Laboratories (Abbott Laboratories, Abbott Park, IL, USA).

2. Experimental

2.1. Equipment

The HPLC system consisted of a Model SP8800 ternary pump (Spectra-Physics, San Jose, CA, USA), Model RF551 fluorimetric detector (Shimadzu Scientific Instruments, Columbia, MD, USA), and Model SP8880 autosampler (Spectra-Physics). The detector output was digitized and data processed using a TurboChrom TC3 data system (PE-Nelson Analytical, Cupertino, CA, USA). C_8 Bond-Elut solid-phase extraction cartridges (Varian, Harbor City, CA, USA) and the Vac-Elut SP 24 apparatus (Varian) were used to extract the analytes from plasma and serum.

2.2. Reagents

Sertindole, internal standard (Lu 26-009) and all potential metabolites of sertindole (Fig. 2) were provided by H. Lundbeck A/S (Copenhagen, Denmark). Methanol, acetonitrile, tetrahydrofuran, and isopropyl alcohol were HPLC grade (Fisher Scientific, Fairlawn, NJ, USA). Reagent-grade ammonium hydroxide (28%) was also obtained from Fisher Scientific.

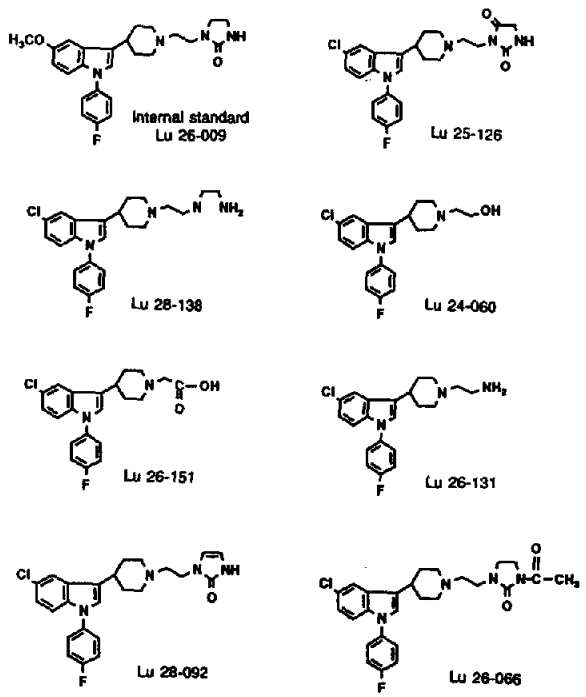

Fig. 2. Structures of Lu 26-009 (internal standard) and potential sertindole metabolites.

Ethanol, U.S.P. (200 proof), was obtained from Asper (Shelbyville, KY, USA).

2.3. Extraction procedure

The internal standard (50 μl of a 10 ng/ml stock solution in ethanol) was added to 1 ml of plasma and vortex-mixed. Sample mixtures were then loaded onto C_8 solid-phase extraction cartridges which had been preconditioned by rinsing with 2×2 ml of acetonitrile, 3×3 ml of methanol, and 2×2 ml of distilled water to wash out any potentially pre-existing chromatographic interferences. Thereafter, the cartridges were sequentially washed with 3×2 ml of distilled water and 3×1 ml of acetonitrile. The analytes were then eluted using 2×1 ml of methanol. The eluates were evaporated to dryness at 30°C under nitrogen and the residues were reconstituted in 200 μl of the mobile phase and 190 μl was injected onto the column.

2.4. Chromatographic conditions

The mobile phase consisted of *n*-hexane–isopropyl alcohol–methanol–tetrahydrofuran–28% ammonium hydroxide (73.6:15:9.6:1.3:0.65, v/v). Before use, the mobile phase was filtered through a 0.2-μm nylon membrane (Alltech Associates, Deerfield, IL, USA) and then thoroughly degassed under vacuum. During use, the mobile phase was continuously sparged with a gentle stream of helium. The mobile phase was delivered at a flow-rate of 1.0 ml/min at room temperature through a Spherisorb silica normal-phase column (5 μm, 250 × 4.6 mm I.D., Alltech) equipped with a guard column (20 × 2 mm I.D., packed with Partisil 20, Whatman, London, UK). Excitation and emission wavelengths of the fluorimetric detector were set at 260 and 340 nm, respectively.

2.5. Calibration standards

Stock solutions of sertindole were prepared in ethanol at a concentration of 1 mg/ml. This solution was serially diluted with ethanol to provide sertindole solutions at 40, 1.0, and 0.1 μg/ml. A standard solution of sertindole in plasma (8 ng/ml) was prepared by diluting the 0.1 μg/ml solution with pooled normal human plasma. Standards for calibration curves in plasma (0.025, 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 ng/ml) were prepared by serial dilution of the 8 ng/ml plasma stock solution. In a similar manner, a separate set of standards in plasma was prepared independently for use as quality control (QC) samples.

2.6. Quantitation

The calibration standards and QC samples were carried through the analysis along with unknown samples. Analyte/internal standard ratios of the observed peak heights from the calibration standards were subjected to weighted ($1/[\text{concentration}]^2$) linear regression to derive the calibration curves; the concentrations for the unknowns and the QC samples were derived from the curve. The QC results provided additional data for the assessment of assay integrity.

2.7. Analytical variables

The absolute extraction recovery of sertindole from human plasma was estimated using plasma samples spiked at concentrations of 0.071, 0.514 and 2.156 ng/ml. These samples were extracted as described in section 2.3 except that the internal standard was added to the collected extract. In addition, a set of drug-free normal human plasma samples was also extracted; each drug-free plasma extract was then supplemented with sertindole (0.025, 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 ng/ml) along with the internal standard, and was analyzed to construct a calibration curve. The concentrations of the spiked plasma samples were calculated from the curve and compared to the theoretical values to derive the extraction recovery.

The potential metabolites of sertindole were injected onto the HPLC system at a concentration of 2 μg/ml to assess possible chromatographic interferences with sertindole and the internal standard. To assess the intra- and inter-assay precision [coefficients of variation (C.V.)] and accuracy, three QC samples (concentrations at 0.1, 0.65, 3.3 ng/ml) were analyzed using the proposed method on four separate days, and each QC sample was assayed in replicates of six within each run. Using another set of QC samples, the stability of sertindole in plasma and serum was evaluated under light and dark conditions at room temperature, after three repeated freeze–thaw cycles, and after frozen storage.

3. Results and discussion

3.1. Method optimization

Mobile phase

The mobile phase was a modification of that used by the scientists at Shionogi Pharmaceutical. It was composed of *n*-hexane–isopropyl alcohol–methanol–28% ammonium hydroxide–

tetrahydrofuran (100:25:5:0.7:2, v/v). Exploratory experiments indicated that the ratio of *n*-hexane and methanol controlled the retention times of sertindole and the internal standard. The presence of tetrahydrofuran and isopropyl alcohol enhanced the fluorescence intensity of sertindole. Ammonium hydroxide, on the other hand, affected the peak shape of sertindole, probably by altering the pH of the mobile phase. Sertindole had maximal fluorescence at the excitation and emission wavelengths of 260 and 340 nm, respectively.

Detector

Three different models of fluorimetric detectors were evaluated under HPLC conditions: 980 programmable fluorescence detector (Applied Biosystem, Foster City, CA, USA); Spectra System FL2000 (Spectra-Physics); and RF551 (Shimadzu Scientific Instruments). Of these, the RF551 Model provided the greatest sensitivity for sertindole in terms of peak height to noise ratio.

Extraction methods

Our exploratory studies showed that sertindole, the internal standard, and Lu 28-092 (one of the potential metabolites) were retained on the short reversed-phase C_8 column (3 ml, 300 mg) even after elution with 6 ml of acetonitrile. In contrast, at least 80% of sertindole and the internal standard was eluted from the same column using only 1 ml of methanol. This high solvent selectivity was the key to the success of our extraction method, as most plasma contaminants could be removed by elution with acetonitrile, thus avoiding chromatographic interference. Sufficient methanol was then used to elute the analyte and the internal standard to achieve near-complete recovery. To our knowledge, there have been no compounds reported to have such high selectivity between acetonitrile and methanol on this C_8 extraction cartridge. The proposed solid-phase approach is very rapid, selective, and efficient, requiring only about 1 h for an analyst to complete the preparation of 60 samples.

HPLC columns

Spherisorb silica normal-phase columns manufactured by Phenomenex (5 μm, 250 × 4.6 mm I.D., Phenomenex, Torrance, CA, USA) and Alltech (same dimensions) were examined under identical chromatographic conditions. For unknown reasons, the Phenomenex column failed to provide baseline separation for sertindole and the internal standard. Thus, the Alltech column was chosen for assay validation.

Guard columns

Guard columns, such as Waters Nova-pack silica (Waters Chromatography Division; Millipore, Milford, MA, USA) and Phase Sep normal-phase (Phase Separation, Norwalk, CT, USA), were evaluated along with a column packed with Partisil 20 (Whatman, Clifton, NJ, USA). The Waters guard column gave a large and broad unknown chromatographic peak between 6 and 9 min that co-eluted with several potential metabolites of sertindole. The Phase Sep guard column yielded wide and split peaks for both sertindole and the internal standard. The results from the guard column packed with Partisil 20 were the most satisfactory.

3.2. Performance of chromatographic system

Fig. 3 shows a representative chromatogram of blank plasma and calibration plasma containing 1 ng/ml sertindole. Pooled normal human plasma yielded relatively clean chromatograms with no significant interfering peaks. Both sertindole and the internal standard showed sharp, well-defined peaks at retention times of 12.0 and 13.1 min, respectively, with baseline separation. The chromatographic peaks due to potential metabolites were well resolved from those of sertindole and the internal standard; retention times are listed in Table 1. Because of the relatively short retention times of sertindole and the internal standard, each chromatographic run required approximately 15 min. This made it possible to analyze about 60 clinical samples per day, including those used for standard curves and quality controls.

To assess the possible differences between

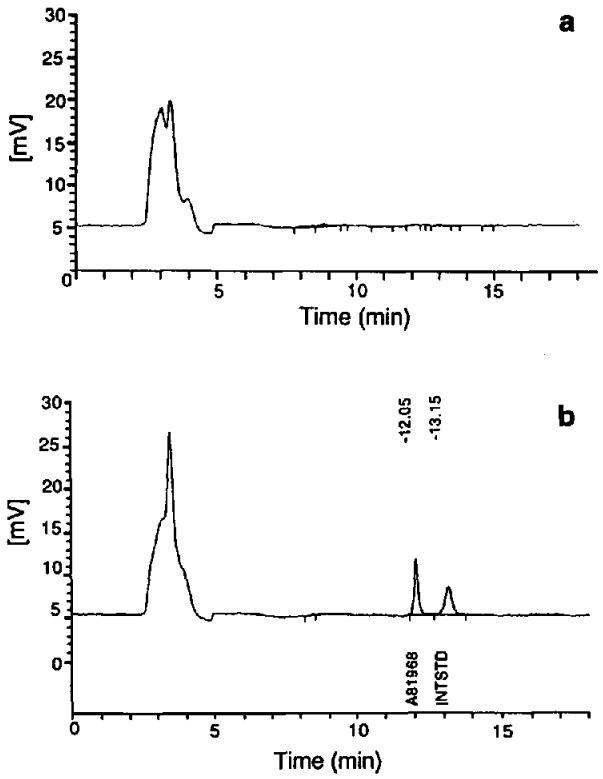

Fig. 3. (a) Chromatogram of blank pooled human plasma. (b) Chromatogram of pooled human plasma spiked with a sertindole concentration of 1 ng/ml. A81968 = sertindole, INTSTD = internal standard.

serum and plasma drug concentrations, blood samples were individually collected into heparinized and non-heparinized tubes from four volunteers (two male and two female), and were spiked with sertindole (three levels from 0.93 to 93 ng/ml). The serum and plasma samples were harvested and subjected to the proposed assay. The results showed no significant differences between plasma and serum concentrations from whole blood spiked with an equivalent amount of sertindole. These results indicate that human serum and plasma samples can be analyzed interchangeably, with no apparent effects associated with donor's gender.

Cleanliness of the guard column was essential to maintain sharp and symmetrical peak shapes for sertindole and internal standard as well as good performance of the system. This was achieved by changing the guard column packing material daily.

Table 1
Retention times of some potential sertindole metabolites

Compound	Retention time (min)
Lu 28-138	–[a]
Lu 26-151	–[a]
Lu 25-126	7.36
Lu 24-060	7.97
Lu 26-066	8.32
Lu 28-092	8.54
Lu 26-131	17.79

[a] No peaks showed in the chromatogram, even when a large amount (400 ng on column) was injected onto the HPLC system.

3.3. Calibration curve and sensitivity

During the validation study, calibration curves were generated over a sertindole concentration range of 0.025 to 4 ng/ml. The curves were all linear with mean coefficient of determination of 0.9919 (S.D = ± 0.0069, $n = 4$). In a separate experiment, good linearity was also obtained in the range of 0.025 to 8 ng/ml. The lower limit of quantitation was 0.025 ng/ml with an associated C.V. of 5.9% (detailed data not shown here). Using a peak-to-noise ratio of 3 as a criterion, the estimated lower limit of detection was 0.01 ng/ml.

3.4. Recovery

As shown in Table 2, the absolute recovery of sertindole was almost quantitative, being more than 89% over a 30-fold concentration range. The recoveries were consistent from sample to sample as evidenced by C.V.s of $< 3\%$ for the high and medium levels and of $< 10\%$ for the low level. Recovery was apparently not concentration-dependent; this was also reflected in good linearity of the calibration curves.

3.5. Accuracy and precision

Intra- and inter-assay precision and accuracy are illustrated in Table 3. Intra-assay C.V.s were

Table 2
Absolute recoveries of sertindole from pooled normal human plasma using solid-phase extraction technique

Sample	Recovery (ng/ml)		
No.	0.071 ng/ml	0.514 ng/ml	2.156 ng/ml
1	0.068	0.454	1.950
2	0.085	0.479	1.906
3	0.072	0.481	1.925
4	0.078	0.448	1.927
5	0.067	0.465	1.958
6	0.075	0.476	1.871
Mean ± S.D.	0.074 ± 0.007	0.467 ± 0.014	1.923 ± 0.032
C.V. (%)	9.13	2.93	1.64
Recovery (%)	104.2	90.9	89.2

All calculations were performed prior to rounding.

relatively low, with values within 5.2% at concentrations of 0.664 and 2.656 ng/ml and within 11.7% at a concentration of 0.133 ng/ml. The inter-assay C.V. values at concentrations of 0.133, 0.664, and 2.656 ng/ml were 10.5, 5.8, and 7.3%, respectively. The percentage deviation of the mean assayed concentrations from the target values ranged from −0.8 to 24.4%, −1.0 to 13.2%, and −5.5 to 9.6% at concentrations of 0.133, 0.664, and 2.656 ng/ml, respectively. These data indicate good intra- and inter-assay accuracy of the method.

Table 3
Accuracy and precision of sertindole assay

	Concentration (mean ± S.D.) (ng/ml)	*n*	C.V. (%)	D.T.[a] (%)
Concentration = 0.133 ng/ml				
Assay 1	0.132 ± 0.013	6	10.2	−0.8
Assay 2	0.166 ± 0.019	6	11.7	24.4
Assay 3	0.137 ± 0.007	6	4.9	3.3
Assay 4	0.155 ± 0.003	6	2.2	16.5
Inter-assay	0.147 ± 0.016	4	10.5	10.9
Concentration = 0.664 ng/ml				
Assay 1	0.692 ± 0.006	6	0.8	4.3
Assay 2	0.680 ± 0.023	6	3.3	2.4
Assay 3	0.657 ± 0.021	6	3.1	−1.0
Assay 4	0.751 ± 0.014	6	1.8	13.2
Inter-assay	0.695 ± 0.040	4	5.8	4.7
Concentration = 2.656 ng/ml				
Assay 1	2.509 ± 0.088	6	3.5	−5.5
Assay 2	2.523 ± 0.131	6	5.2	−5.0
Assay 3	2.557 ± 0.047	6	1.8	−3.7
Assay 4	2.911 ± 0.060	6	2.0	−9.6
Inter-assay	2.625 ± 0.192	4	7.3	−1.2

[a] DT = Difference from target.

3.6. Stability

Sertindole was stable in serum or plasma at room temperature for at least 48 h under either light or dark conditions. No degradation in either serum or plasma was observed at −20°C over a period of 28 days. In addition, sertindole appeared to be stable after three repeated freeze–thaw cycles, as evidenced by percent changes from target concentration of only −4.6% and 6.5% for plasma and serum, respectively.

3.7. Assay application

This analytical method has been successfully applied to clinical samples in Phase I pharmacokinetic studies. No interferences from endogenous compounds or metabolites of sertindole have been found. A typical chromatogram from a Phase I study is shown in Fig. 4. The serum samples from this multiple dosing study were diluted 1:10 in pooled drug-free human plasma before preparation for the assay. An additional quality control set at 66.4 ng/ml sertindole was diluted and assayed. Both inter-assay and intra-assay variabilities for the diluted quality control samples were within 7.1%. The percentage differences between the mean assayed concentrations and the target concentrations ranged from 0.1% to 5.9%, indicating a high degree of accuracy. As shown in Fig. 4, at least two additional metabolite peaks were found that were well resolved from sertindole and the internal standard. The peak occurring at 8.34 min (identified as peak 1) which co-eluted with Lu 28-092 was further confirmed to be Lu 28-092 by using liquid chromatography–mass spectrometry. No information about peak 2 occurring at 9.2 min was available. Fig. 5 shows the serum concentration–time profile of sertindole in samples collected from a healthy subject after the last dose in a multiple-dose Phase I study. A detailed analysis of the pharmacokinetics of sertindole will be presented elsewhere.

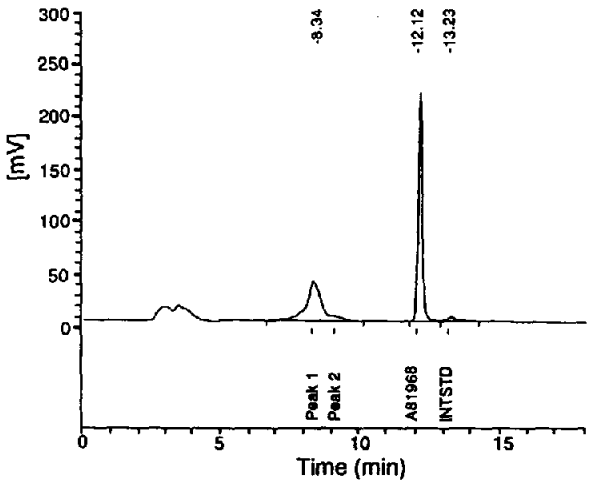

Fig. 4. Representative chromatogram of serum sertindole sample in a healthy volunteer. A81968 = sertindole, INTSTD = internal standard.

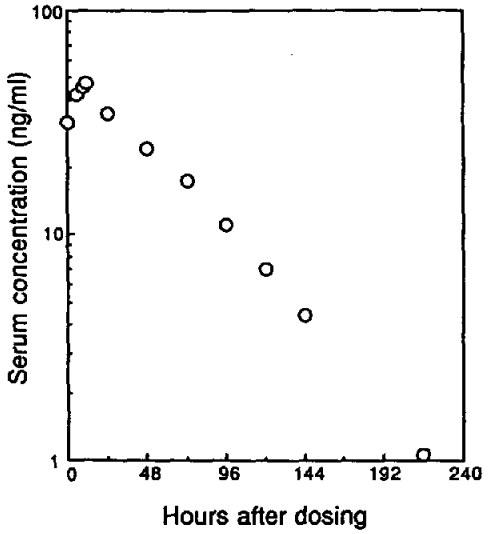

Fig. 5. Serum sertindole concentration–time profile in a healthy volunteer.

4. Conclusions

A new method for the sensitive, specific, rapid, and robust determination of sertindole in human plasma and serum was accomplished using normal-phase HPLC and fluorometric detection. This solid-phase extraction method offers both ease of sample preparation and high recovery, resulting in high sensitivity and excel-

lent reproducibility. The method has proven suitable for use in pharmacokinetic studies of sertindole.

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