Development and validation of a reverse phase High Performance Liquid Chromatographic method, using standard addition calibration, for determination of risperidone in human plasma

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Abstract

Risperidone is a drug of choice in the management of psychosis and high performance liquid chromatography (HPLC) is regarded as the most sensitive and accurate method for quantitative analysis of risperidone, as well as other drugs having low plasma concentrations in biological fluids. A simple and accurate HPLC method was developed for the determination of risperidone in human plasma using the standard addition calibration technique to augment the weak signals due to low plasma concentrations of risperidone. Risperidone was eluted using isocratic mode with a mobile phase of methanol: acetonitrile (60:40) on a Chemisil ODS C18 column (250 mm×4.6 mm i.d., 5 µ particle size). The sample injection volume, flow rate, column temperature and wavelength of detection were set at 10 µl, 1.0 ml/min, 35 ˚C and 280 nm respectively. The run time for the method was 7 minutes, with risperidone having a retention time of 3.566 minutes. The method was linear within the concentration 10-50 ng/ml (R^2= 0.998), with LOD and LOQ of 0.13 and 0.40 ng/ml respectively. The precision and relative error was 3.44 % and 3.38 % respectively. The developed method can be employed in the quantitative determination of risperidone in pharmacokinetic and bioequivalence studies.

Keywords: Risperidone; High-performance liquid chromatography; Standard addition; Plasma
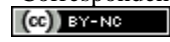
INTRODUCTION

Risperidone (Figure 1), 3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl] ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido -[1,2-a]-pyrimidin-4-one belongs to the chemical class of benzisoxazole derivatives and it is regarded as the drug of first choice in the management of schizophrenia and mania [1]. Several methods have been developed for the determination of risperidone in plasma. However, the reverse phase high performance liquid chromatographic (RP-HPLC) methods are the most widely employed because of its simplicity, versatility and range of compounds of a diverse polarity and molecular mass they are able to handle [2]. The quantitative detection of risperidone in plasma has primarily been achieved using HPLC coupled

to various detectors. Initially, the use of electrochemical detection was employed but this method was plagued by the drifting of the electrode response with time, requiring the frequent calibration with standards [3-6]. Later on, mass spectrometric (MS) detection (single stage and tandem) was introduced, which has improved sensitivity and specificity [7-11]. However, HPLC with MS detection is expensive and not readily available. This has limited its use, especially in developing countries, thus promoting the use of HPLC with an ultraviolet (UV) detector, which is often more convenient and readily available [9]. Oral doses of risperidone ranges from 2- 8 mg per day for the treatment of chronic schizophrenia and at steady state, these doses result in low plasma levels in the ng/ml range [5]. Earlier methods for the quantification of risperidone and 9-hydroxyrisperidone using HPLC with UV detection [12] may be suited for therapeutic drug monitoring of target concentrations. However, their lower levels of quantification are not particularly suited for pharmacokinetic studies [11]. The aim of this study was to develop a RP-HPLC method for the determination of risperidone with UV detection that can be applied in pharmacokinetics study by using the standard addition calibration method in order to augment the weak signals due to the low levels of risperidone in plasma.

EXPERIMENTAL METHODS

Materials. HPLC Agilent 1260 LC System controlled by OpenLab software (Agilent, USA), Chemisil ODS C18 Column (250 mm×4.6 mm i.d., 5µ particle size), risperidone reference standard (Solarbio, China), clozapine reference standard (Solarbio, China), HPLC grade methanol and acetonitrile (Merck, Germany).

Methods. The extraction and HPLC procedures were developed as follows:

Preparation of stock standard solution of risperidone and clozapine. Stock standard solutions equivalent to 100 µg/ml risperidone and clozapine were prepared by dissolving 10 mg of risperidone and clozapine reference standards respectively in 100 ml of methanol. Subsequently, working solutions of 1000 ng/ml of risperidone and 500 ng/ml of clozapine were prepared from their respective stock solutions.

Preparation of blank plasma samples. Whole blood was collected via venipuncture at the forearms in EDTA bottles from a healthy male volunteer. The blood was centrifuged at 4500 rpm for 10 minutes and the supernatant collected in plain collection tubes and stored at ± 20 ˚C. Protein precipitation of 1 ml of the thawed plasma was carried out by adding 5 ml of acetonitrile and centrifuging at 4000 rpm for 5 minutes. The supernatant was injected into the HPLC in order to get the chromatogram of blank plasma.

Sample preparation and extraction. A portion (1 ml) of the blank plasma was spiked with 0.5 ml of 1000 ng/ml of risperidone and 0.5 ml of 500 ng/ml of clozapine, vortex mixed for 5 minutes and precipitated by adding acetonitrile to the 5 ml mark. The mixture was then centrifuged at 4000 rpm for 5 minutes and the supernatant was then injected into the HPLC machine for analysis to produce a chromatogram of risperidone and clozapine in plasma.

Chromatographic conditions. The wavelength of maximum absorption (λ_{max}) was determined for risperidone using the UV spectrophotometer. The λ_{max} would be used as the wavelength of detection for the HPLC method. The chromatographic separation was achieved on a Chemisil ODS C18 column (250 mm×4.6 mm i.d., 5µ particle size). The composition of the mobile phase (methanol: acetonitrile), flow rate, injection volume and column temperature were varied until the conditions were optimized.

Standard addition calibration. The standard addition calibration method was used for analysing the analyte (risperidone) in plasma. This method involves the spiking of a known and fixed concentration of the standard risperidone solution (1000 ng/ml) into each sample that is to be analysed. Aliquots (1 ml) of risperidone standard solutions of the following concentrations: 10, 20, 30, 40 and 50 ng/ml of risperidone were transferred into five separate plain collection tubes and 1 ml of thawed plasma was added to each tube and shaken for 1 minute. A portion (1 ml) of the fixed standard risperidone (1000 ng/ml) and 0.5 ml of 500 ng/ml of clozapine (internal standard) was transferred into each tube and subjected to the extraction procedure developed earlier. The corresponding peak height ratio (I_{X+S}) gotten for each calibrating concentration (10- 50 ng/ml) is substituted in the formula below:

$$\frac{[X]_I}{[S]+[X]_F} = \frac{I_X}{I_{X+S}} \ldots\ldots [13]$$

where $[X]_I$ = Initial concentration of analyte (10, 20, 30, 40 or 50 ng/ml)

$[X]_F$ = Final concentration of analyte in 5 ml solution (2, 4, 6, 8 or 10 ng/ml respectively)

$[S]_F$ = Final concentration of 1000 ng/ml in 5 ml solution (100 ng/ml)

I_{X+S} = Peak height ratio of analyte and spiking concentration of 1000 ng/ml

I_X = Peak height ratio of analyte alone (unknown).

I_X is the unknown and was calculated from the formula above. The corresponding I_X values for each concentration (10-50 ng/ml) were then used to construct a calibration curve.

Method validation.
The RP-HPLC method developed was validated according to ICH guidelines [14].

Linearity: Serial dilutions were made from the stock solution of risperidone to give solutions of 10, 20, 30, 40, 50 ng/ml and a portion (1 ml) of each solution was transferred into five separate plain collection tubes containing and 1 ml of thawed plasma was added to each tube

and shaken for 1 minute. A portion (1 ml) of the fixed standard risperidone (1000 ng/ml) and 0.5 ml of 500 ng/ml of clozapine (internal standard) were transferred into each tube and subjected to the extraction procedure described earlier and injected in triplicate into the HPLC machine.

Accuracy. To ascertain accuracy of the method, a known concentration of risperidone (100 ng/ml) was added to 1 ml of plasma, subjected to the sample extraction procedure described earlier and injected into the HPLC machine. This known concentration was then spiked at three different concentration levels (80, 100 and 120 %) of the known concentration to obtain final concentrations 180, 200 and 220 ng/ml of risperidone respectively. The plasma samples were also subjected to the sample extraction procedure described earlier and injected into the HPLC machine.

Precision. Three different concentrations (10, 30 and 50 ng/ml) were injected thrice (at an hourly interval) into the HPLC machine on the same day and the percentage coefficient of variation (% CV) was calculated to determine intra-day precision. This was repeated on three different days to determine inter-day precision. Prior to this, the sample extraction procedure was carried out.

Specificity. An aliquot (1 ml) of thawed plasma from six subjects were spiked individually with a known concentration of risperidone (150 ng/ml) and clozapine (50 ng/ml). The sample extraction procedure was carried out on the plasma samples and injected into the HPLC machine.

Robustness. The robustness of the method was determined by spiking thawed plasma (1 ml) with risperidone and clozapine to give final concentrations of 100 ng/ml and 50 ng/ml respectively. The sample extraction procedure was carried out on the plasma samples and injected into the HPLC machine. Three chromatographic conditions (mobile phase,

flow rate and wavelength of detection) were varied slightly and the effect of these variations on the retention time and resolution of risperidone was observed.
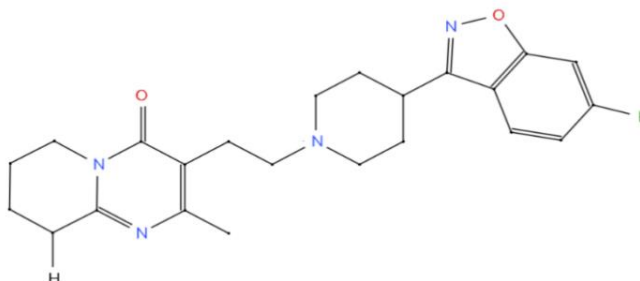

Figure 1: Chemical structure of risperidone

Table 1: Optimized chromatographic conditions of developed RP-HPLC method for the quantitative analysis of risperidone in plasma

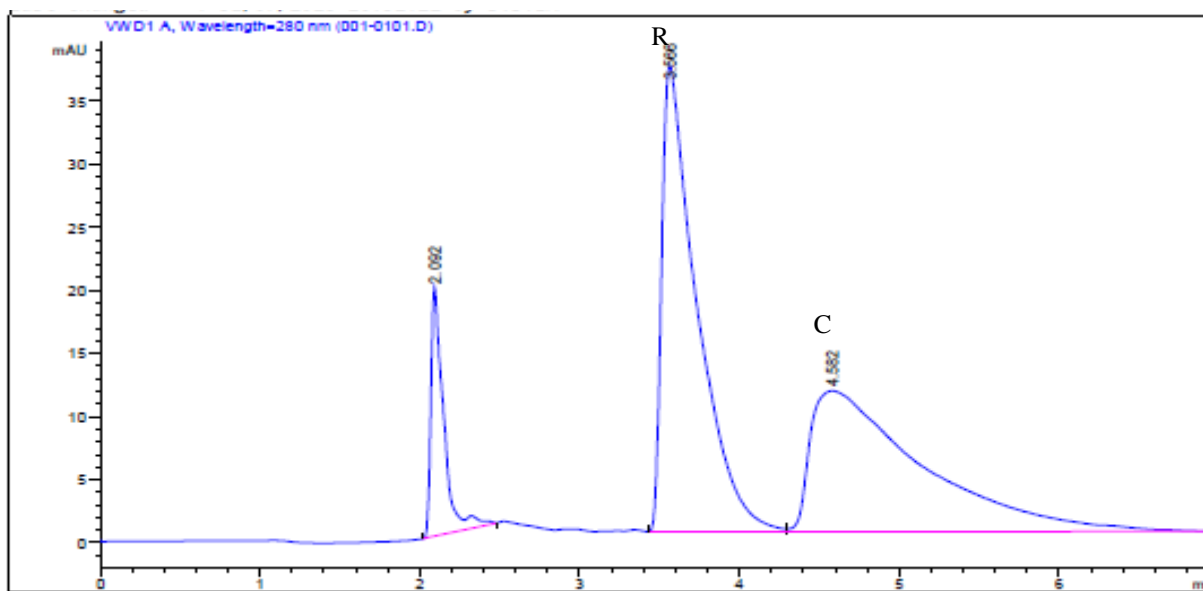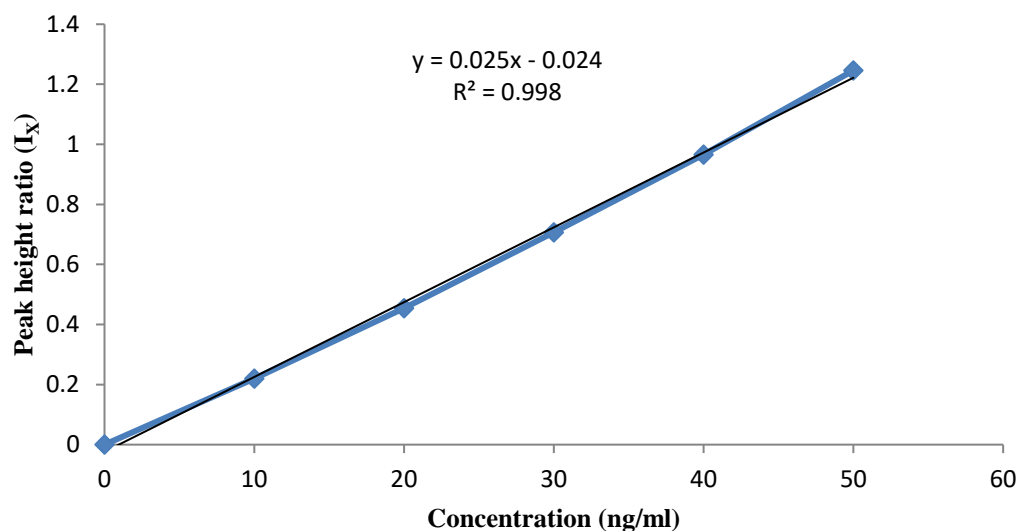| Chromatographic condition	Optimized values/ conditions
Stationary phase (column)	Chemisil ODS C18 (250 mm×4.6 mm i.d., 5µ particle size)
Mode	Isocratic
Mobile phase	Methanol: Acetonitrile (60:40)
Detection wavelength	280 nm
Runtime	7 minutes
Flow rate	1.0 ml/min
Volume of injection	10 µl
Column temperature	35 ˚C

Figure 2: Chromatogram of extract of risperidone and clozapine spiked in plasma obtained from a healthy human showing retention time of risperidone (R) and internal standard (clozapine, C). Resolution of peaks = 2.3

Table 2: Calibration parameters of developed RP-HPLC method for the quantitative analysis of risperidone in plasma

Concentration (ng/ml)	Peak height ratio of analyte and spiking concentration of 1000 ng/ml (I_{X+S})	Calculated peak height ratio of analyte alone (I_X)
10	2.248	0.220
20	2.363	0.455
30	2.499	0.707
40	2.608	0.966
50	2.742	1.246

$y = 0.025x - 0.024$
$R^2 = 0.998$

Figure 3: Calibration curve of developed RP-HPLC method for the quantitative analysis of risperidone in plasma

Table 3: Validation parameters of developed RP-HPLC method for the quantitative analysis of risperidone in plasma

Parameters	Values
Limit of detection (LOD)	0.13 ng/ ml
Limit of quantification (LOQ)	0.40 ng/ ml
Accuracy (percentage recovery)	96.62 %
Accuracy (% E_R)	3.38 %
Precision (% CV)	3.44 %
Specificity (percentage recovery)	94.95 %
Robustness (percentage deviation)	5.45 %

RESULTS AND DISCUSSION

The optimized chromatographic conditions (Table 1) were found to elute risperidone at 3.566 minute (Figure 2). The chromatographic conditions were optimized by varying the flow rate, injection volume, column temperature and the mobile phase. The flow rate was optimized at 1.0 ml/min with a resolution of 2.3 and increasing the flow rate to 1.2 ml/min diminished resolution to < 1, which is similar to what, was reported in earlier research works [10-12]. The United States Food Drug and Administration's Centre for Drug Evaluation and Research (FDA-CDER) guidelines for HPLC, set the acceptable resolution of peaks at >2 [15]. However, some handlers of HPLC have accepted resolution values of between 1.5 to 2.0, especially during separation of complex samples and when tailing occurs [16,17]. Injection volume was optimized at 10 µl, which is an improvement on the 60 µl, and 350 µl employed in previous

research works [12, 18]. This could be attributed to the use of the standard addition calibration method. The mobile phase contains more methanol than acetonitrile, thereby reducing cost and still achieving good resolution and efficiency. The developed method was validated for linearity, precision, accuracy, robustness and specificity according to the ICH 2005 guidelines [14]. Table 2 shows the calibration parameters of the calibration curve. Linearity was observed within the concentration range of 10- 50 ng/ml as indicated by the coefficient of determination (R^2) of 0.998 (Fig 3). The method was found to be highly sensitive as the LOD and LOQ values were 0.13 and 0.40 ng/ml respectively, thus making the developed method suitable for pharmacokinetic studies due to its low levels of detection and quantification [11]. Table 3 outlined the validation parameters of the RP-HPLC method. The average precision at three concentration levels was 3.44 %, which is within the acceptable limit of < 15 %. The percentage recovery was 96.62 % and was within the acceptable range of 95 – 105 %. The relative error (% E_R) was 3.38 %, which also fell within the acceptable range of 1-5 %. The robustness of the method showed that slight variations in the chromatographic conditions would not affect the retention time of the analyte as the percentage deviation of the retention times recorded was 5.45 %. Finally, the specificity study reflected the large inter-individual variability that was described in adult patients during routine therapeutic drug monitoring [5]. However, the recovery for each blank was > 90 %, which is adequate for pharmacokinetic studies [11].

Conclusion. A RP-HPLC method for the determination of risperidone in human plasma with UV detection was developed and validated using the standard addition calibration method. The method is simple, precise, accurate and can be used for pharmacokinetic and bioequivalence studies involving risperidone.

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