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Analysis of cisapride in neonatal plasma using high-performance liquid chromatography with a base-stable column and fluorescence detection

Y. Preechagoon, B.G. Charles*

Department of Pharmacy, Steele Building (Floor 1), The University of Queensland, Brisbane, Queensland 4072, Australia

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Abstract

A simple, selective, sensitive and precise high-performance liquid chromatographic plasma assay for the prokinetic drug cisapride is described. Alkalinised samples of plasma (100 μ l) were extracted with 1.0 ml of 10% (v/v) isopropanol in chloroform, dried, redissolved in mobile phase and injected. Chromatography was performed at 20°C by pumping a mobile phase of acetonitrile (370 ml) in pH 5.2, 0.02 M phosphate buffer (630 ml) at 1.0 ml/min through a C₈ Symmetry column. Cisapride and the internal standard were detected by fluorescence monitoring at 295 nm (excitation) and 350 nm (emission), and were eluted 5 min and 8 min, respectively, after injection. Calibration plots in bovine serum albumin (3% w/v) were linear ($r > 0.999$) from 5 to 250 ng/ml. Intra-day and inter-day precision (C.V.) was 9.5%, or less, and the accuracy was within 5.5% of the nominal concentration over the range 8–200 ng/ml. Total assay recovery was above 82%. Endogenous plasma components, the major cisapride metabolite (norcisapride), and other drugs used in neonatal pharmacotherapeutics did not interfere.

1. Introduction

Cisapride, (\pm)-*cis*-4-amino-5-chloro-N-[1-[3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide monohydrate is a relatively new, orally administered prokinetic agent which is chemically related to metoclopramide, but lacks antidopaminergic and central depressant effects [1–3]. It acts by stimulating acetylcholine release from postganglionic nerve endings of the myenteric (Auerbach's) plexus in gastrointestinal smooth muscle, and by activating 5-HT₄ receptors in the intestinal wall [1,4–6].

Cisapride has been shown to be effective in treating gastro-oesophageal reflux disease in adults, children and neonates, and it shows promise in the management of functional dyspepsia, gastroparesis, intestinal pseudo-obstruction and irritable bowel syndrome [2–9].

Despite the rapidly increasing use of this drug there are no pharmacokinetic data whatsoever in paediatric patients. Such information is important and necessary when attempting to develop appropriate dosage regimens and therapeutic strategies for cisapride, especially in premature babies who are particularly susceptible to reflux disorders. There is only one other published method for cisapride analysis in which the drug is

* Corresponding author.

extracted from plasma followed by reversed-phase chromatography and UV absorbance detection [10]; however, it is totally unsuitable for paediatric applications due to the relatively large volume (2 ml) of sample required.

Here we describe a simple, rapid and robust high-performance (HPLC) method suitable for the determination of cisapride in plasma from neonates. Only 0.1 ml of plasma is required and, for detection, advantage is taken of the native fluorescence of cisapride and the internal standard which provides a high degree of sensitivity and selectivity.

2. Experimental

2.1. Reagents and materials

Cisapride (R 51 619) and the internal standard (R 54 680; *cis*-4-amino-5-chloro-N-[1-[5-(4-fluorophenoxy)pentyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide monohydrate), were kindly donated by Janssen-Cilag (Sydney, NSW, Australia). Acetonitrile, methanol and chloroform (Merck, Darmstadt, Germany) were HPLC grade. 2-Propanol was purchased from Mallinckrodt Australia (Clayton, Vic., Australia). Bovine serum albumin (BSA, Fraction V) was obtained from Sigma (St. Louis, MO, USA). Potassium dihydrogen orthophosphate, orthophosphoric acid (85%) and sodium hydroxide were analytical reagent grade and were purchased from BDH Chemicals Australia (Kilsyth, Vic., Australia). Freshly distilled water, filtered through a 0.45 μm membrane, was used to make up all aqueous reagents.

2.2. Instrumentation

The chromatographic system comprised a Model LC-10AS pump, a Model RF-535/535T fluorescence detector, both supplied by Shimadzu (Kyoto, Japan) and a Model 7125 injector (Rheodyne, Cotati, CA, USA). Separations were performed on an octyl (C_8) Symmetry column (150 mm \times 3.9 mm I.D., 5 μm particle size) obtained from Waters Australia

(Sydney, NSW, Australia). Chromatograms were recorded on a Model 111 strip-chart, analog recorder (Kipp and Zonen, Delft, Netherlands).

2.3. Standards and controls

Master stock solutions (0.1 mg/ml) of cisapride and internal standard in methanol were prepared monthly and kept tightly sealed at -20°C . Working standards of both compounds were obtained by dilution to 1 $\mu\text{g}/\text{ml}$. Standards were freshly prepared by supplementing 3% w/v BSA with the cisapride working standard solution to give concentrations of 250, 150, 100, 50, 20, 10 and 5 ng/ml. Controls containing 200, 40 and 8 ng cisapride per ml were also prepared in 3% aqueous BSA, and stored at -70°C .

2.4. Sample preparation

Standards, controls, or unknown plasma samples (100 μl) were pipetted into 10 ml glass test tubes, alkalised with 70 μl of 1.0 M sodium hydroxide and extracted by vortex-agitation for 2 min with 1.0 ml of 2-propanol (10% v/v) in chloroform containing 30 ng of the internal standard. The tubes were centrifuged at 1800–1900 g for 1 min. The organic layers were carefully decanted into a second series of 10-ml glass tubes which were placed in a heating block (60°C for 10 min) and then evaporated under a gentle stream of air supplied through an overhead manifold. Each of the dried extracts was dissolved in 100 μl of mobile phase and injected.

2.5. Chromatography

All HPLC work was performed in an air-conditioned laboratory at $20 \pm 3^\circ\text{C}$. Separations were conducted using a mobile phase of acetonitrile (370 ml) plus 0.02 M, pH 5.2 phosphate buffer (630 ml), and pumped at a flow-rate of 1.0 ml/min. The mobile phase was filtered through a 0.45- μm pore-size membrane filter and ultrasonically degassed before use. Peaks were measured fluorometrically at excitation and emission wavelengths of 295 nm and 350 nm, respectively.

The speed of the recorder was 2 mm/min and the total run time was 10 min.

2.6. Quantitation

Calibration curves were obtained by weighted regression [11] of the peak-height ratios of cisapride to internal standard, on cisapride concentration in the standards. Unknown cisapride concentrations were calculated by inverse prediction from the calibration equation. Standards, controls, and patient specimens were analysed identically.

2.7. Recovery

Absolute recoveries of 200, 40 and 8 ng/ml concentrations of cisapride in 3% BSA were determined by assaying these samples ($n = 10$) as described above and comparing the peak heights of both cisapride and internal standard with those obtained from direct injection of the compounds dissolved in mobile phase.

2.8. Precision and accuracy

Within-day precision was determined by analysing the three spiked controls 10 times over one day in no fixed order, while between-day precision was determined from the analysis of each control once on each of 10 different days. Assay precision (C.V.) was assessed by expressing the standard deviation of the measurements as a percentage of the mean value. The accuracy of the assay was estimated for each seeded control by comparing the target concentration of cisapride in BSA with the assayed concentration.

2.9. Stability

Stability was determined in samples comprising 250 ng/ml and 20 ng/ml of cisapride in 3% BSA solution under the following conditions: samples stored at -70°C for 8 weeks; processed samples left at room temperature ($20 \pm 3^{\circ}\text{C}$) over 24 h; samples stored at room temperature ($20 \pm 3^{\circ}\text{C}$) over 6 h, in the refrigerator (ca. 4°C)

over 6 h, and in the freezer compartment of the refrigerator (ca. -20°C) over 48 h.

2.10. Selectivity

The selectivity of the assay was examined in relation to interference from endogenous substances in drug-free neonatal plasma, and a major cisapride metabolite, norcisapride [12]. The retention times of norcisapride, and a selection of drugs used in neonatology were measured following injection of solutions of these compounds in mobile phase.

3. Results and discussion

Base-stable HPLC columns show promise for the reversed-phase chromatography of basic molecules (such as cisapride) and obviate the need to add triethylamine or diethylamine to improve peak shape. Fig. 1 shows chromatograms of drug-free neonatal plasma, 3% aqueous BSA containing 250 ng/ml cisapride, and plasma containing 58.5 ng/ml from blood drawn 4 h after an oral cisapride dose of 0.2 mg/kg to a newborn infant (weight 3400 g). Cisapride and the internal standard were eluted as sharp, symmetrical peaks after 5 and 8 min, respectively, in areas of the chromatogram free from endogenous peaks. Compound R 54680 served as a suitable internal standard since it has similar stability, detectability and chromatographic properties to cisapride. The retention time of cisapride could be altered markedly by adjustment of the pH of the buffer in the mobile phase. The optimal pH was 5.2, but at lower pH values cisapride was eluted more quickly and merged with peaks from endogenous substances in plasma. The major metabolite, norcisapride, was eluted after 2 min among extraneous peaks near the solvent front. No effort was made to include this metabolite in the analysis since it has only about one sixth the pharmacological activity of the parent drug [12]. The specificity of the assay was further confirmed by the absence of co-eluting peaks when the following drugs were dissolved in mobile phase and injected: amox-

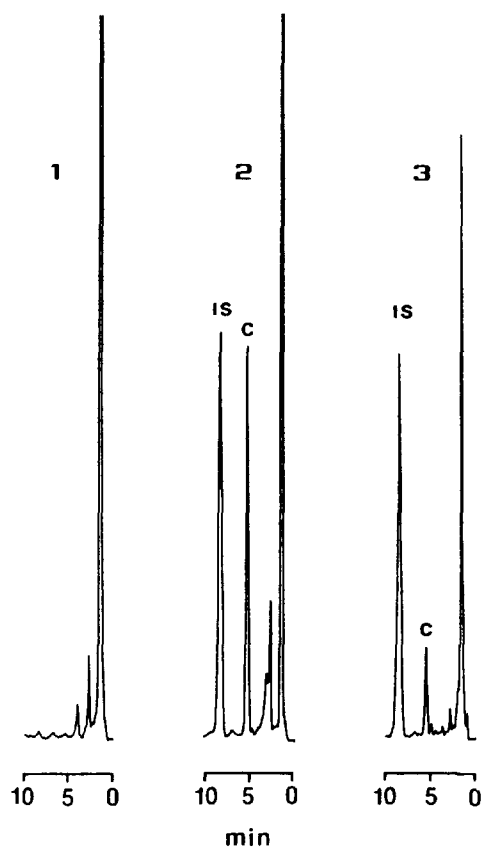


Fig. 1. Chromatograms of (1) drug-free neonatal plasma, (2) 3% BSA (w/v) containing 250 ng/ml cisapride, and (3) plasma sample containing 58.5 ng/ml of cisapride from blood drawn 4 h after a 0.2 mg/kg oral dose of cisapride suspension to a premature neonate. Peaks: C = cisapride; IS = internal standard (R 54680). Fluorescence detector settings: sensitivity range = medium; attenuation = 64.

ycillin, gentamicin, hydrocortisone, dexamethasone, furosemide, indomethacin, metoclopramide, midazolam, theobromine, theophylline, caffeine, and tolazoline. Furosemide was detected by fluorescence and was eluted between cisapride and internal standard at 6 min, but was well-resolved from these compounds. The remaining drugs were either not detected, or were eluted in less than 2 min after injection.

The mean (\pm S.D.) regression equation from 16 replicate calibration curves on different days: $y = (0.00382 \pm 0.00029)x + (0.00955 \pm 0.00426)$ (where, y = peak-height ratio, x = cisapride concentration), showed significant linearity ($r =$

0.9998 ± 0.00021), with statistically insignificant ($p > 0.05$) nonlinear elements in the residual sums of squares, as determined by analysis of variance. Precision and accuracy (8–200 ng/ml) were investigated by replicate analyses of spiked controls (Table 1), and in all cases the within-day and between-day precision was acceptable at a coefficient of variation of 9.5%, or less. In addition, accuracy was within 5.5% when assay results were compared with target concentrations across this range. The limit of quantitation (LOQ) was 8 ng/ml, the cisapride concentration for which the precision approached the acceptable limit of 10% (C.V.). The limit of detection (LOD) defined as the concentration for which peak height was 3 times average baseline noise was 5 ng/ml with 100 μ l of plasma, although there is scope for this to be substantially decreased if larger volumes of plasma are used. Satisfactory assay sensitivity was assisted by high, reproducible recoveries for cisapride and the internal standard as shown in Table 2.

Cisapride and the internal standard were stable when extracted samples (20 and 250 ng/ml plasma) were reconstituted in mobile phase and stored at ambient temperature ($20 \pm 3^\circ\text{C}$) over 24 h. Thus, the HPLC analysis can be conducted unattended when an autosampler is used, for example, when large numbers of samples from a clinical study are to be chromatographed over-

Table 1
Precision and accuracy of HPLC cisapride analysis

Cisapride concentration (ng/ml)			Accuracy ^a (%)
Target	Found (mean \pm S.D.)	C.V. (%)	
Intra-day ($n = 10$)			
200	201.6 \pm 6.7	3.3	0.8
40	41.3 \pm 1.9	4.6	3.2
8	7.8 \pm 0.6	7.7	2.5
Inter-day ($n = 10$)			
200	200.1 \pm 8.8	4.4	0.05
40	42.2 \pm 2.0	4.7	5.5
8	8.4 \pm 0.80	9.5	5.0

^a Defined as: [(measured conc. – target conc.)/target conc.] \cdot 100%.

Table 2
Absolute recoveries of cisapride and internal standard

Concentration (ng/ml)	Recovery (mean \pm S.D., $n = 10$) (%)	
	Cisapride	Internal standard
200	82.2 \pm 7.7	89.1 \pm 5.0
40	86.9 \pm 11.7	93.8 \pm 7.1
8	85.7 \pm 10.3	89.7 \pm 5.9

night. Cisapride was stable in plasma stored either at room temperature, or 4°C for at least 6 h, and at -20°C for at least 48 h. Satisfactory stability was found during storage of at least 8 weeks at -70°C which, in a clinical study, permits stockpiling of samples during collection periods for subsequent batch analysis.

It is difficult to obtain suitable amounts of drug-free plasma from neonates, particularly the very premature babies, due to their unstable clinical state and small blood volumes [13,14]. Moreover, these infants are invariably administered penicillins, cephalosporins, aminoglycosides, or other drugs immediately after birth. For these reasons aqueous 3% BSA was used as the matrix for cisapride standards and spiked controls. The peak-height ratios of cisapride to internal standard were similar when both were extracted from 2 to 5% BSA solutions, the range of serum albumin concentrations commonly encountered in neonatal plasma [15]. Further, the slopes of calibration plots prepared in pooled neonatal plasma and 3% w/v BSA were similar. BSA has been used by us before as a satisfactory matrix in the HPLC analysis of furosemide [16] and dexamethasone [17] during pharmacokinetic studies in neonates.

4. Conclusion

We have described a simple, robust, accurate and precise HPLC method for the analysis of cisapride in plasma. This is one of the first reported clinical applications of the new base-stable, HPLC columns in neonatal medicine. The specificity and sensitivity afforded by fluo-

rescence detection and the short chromatographic run time are features which make this method useful for cisapride monitoring and for other clinical applications including pharmacokinetic studies in premature neonates and older patients.

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