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Journal of Chromatography B, 806 (2004) 263–269
JOURNAL OF CHROMATOGRAPHY B
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Liquid chromatographic determination including simultaneous "on-cartridge" separation of ranitidine cisapride drug combinations from paediatric plasma samples using an automated solid-phase extraction procedure

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Received 12 January 2004; received in revised form 5 April 2004; accepted 6 April 2004

Abstract

HPLC methodology was investigated for the simultaneous determination of cisapride and ranitidine in small volume paediatric plasma samples. Such a simultaneous determination proved difficult due to the small sample volumes, the low concentrations of the drugs and the different log *P* values of the two compounds. The two drugs and their respective internal standards were separated "on-cartridge" using HLB Solid Phase Extraction cartridges and the samples quantified by individual HPLC methodologies. The technique has been applied successfully to 60 paediatric plasma samples containing both cisapride and ranitidine.

Keywords: Ranitidine; Cisapride

1. Introduction

As part of our continuing work into the unlicensed and off label use of drugs in children we have conducted a study involving Ranitidine and Cisapride. Ranitidine is a H_2 receptor agonist and cisapride is a prokinetic agent (see Fig. 1 for structures) and both drugs have been used either separately or in conjunction in the treatment gasto-oesophagal reflux disease [1] in paediatric patients. These two drugs have been used in an unlicensed or off-label fashion within the paediatric population. Due to serious problems with respect to the prolongation of Q_T intervals cisapride has recently been withdrawn from use [2].

The analysis of ranitidine in human plasma is frequently performed using HPLC [3–14]. Although HPLC methods for the determination of plasma ranitidine concentrations vary widely, isocratic reverse-phase HPLC using a μ-Bondpack C18 packed analytical column appears to be the most common mode selected [3,4,9,10,12,13]. Chromatographic separation of ranitidine from plasma constituents has been achieved using a wide variety of mobile phase conditions, however, in more recent years a phosphate-buffered acetonitrile mobile phase has frequently been chosen [3,7–9,12,14]. Despite such a wide variety of HPLC methods it is only recently that a method suitable for the analysis a paediatric plasma samples has been reported [15].

The earliest report of the determination of cisapride in plasma was by Woestenborghs et al. [16] who employed HPLC with UV detection. Several similar methods have been reported, however, these are unsuitable for the analysis of paediatric plasma samples as they require large plasma

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doi:10.1016/j.jchromb.2004.04.007

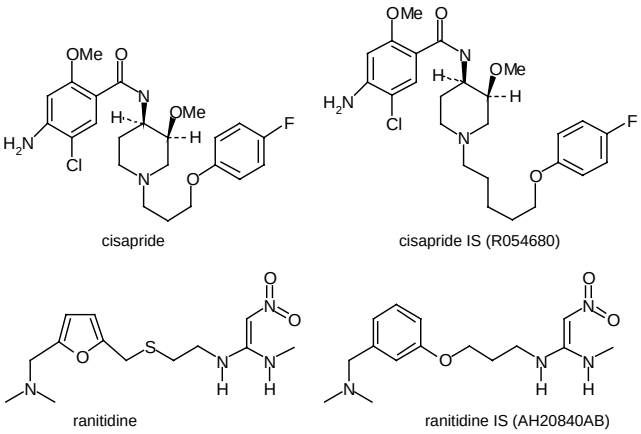

Fig. 1. The structures of cisapride and ranitidine and their corresponding internal standards.

volumes [17,18]. Two methods have been applied to the determination of cisapride in paediatric plasma samples. Preechagoon and Charles [19] describe a HPLC method with fluorescence detection requiring the use of 100 μL of plasma and Cisternino et al. [20] who utilised HPLC with UV detection and 150 μL of plasma. Both these methodologies have been applied to the analysis paediatric samples.

Despite the numerous publications concerned with the co-administration of ranitidine and cisapride there are no reported assays where the two drugs have been determined in the same assay. Rowbotham et al. [21] have investigated the effect of single doses of cisapride and ranitidine, administered simultaneously, on the plasma concentrations of cisapride and ranitidine. In this work the subjects were healthy adult volunteers from whom 10 mL of blood was taken. Plasma obtained from these blood samples was analysed using specific assays for the individual drugs. In the analysis of paediatric samples plasma volumes are often 200 μL or lower and thus new methodologies need to be developed for the quantification of low levels of drugs in such samples. This present paper describes the use of an automated solid phase extraction procedure for the simultaneous separation of cisapride and ranitidine from paediatric plasma samples and their subsequent determination by previously developed individual assays.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade except where otherwise stated. All water was HPLC grade and was obtained using a Millipore-Q Reagent System (Waters, England). Ranitidine hydrochloride was purchased from ICN Biomedicals Inc. (Ohio, USA). AH20480, *N*-[3-[5[[(dimethylamine)-methyl]-fenoxy]propyl]]-*N*′-methyl-2-nitro-1,1-ethenediamine, used as the internal standard in the ranitidine assay, was kindly donated by GlaxoWellcome (Stevenage, UK). Cisapride and its corresponding internal standard R054680 were kindly donated by the Janssen Research Foundation (Beerse, Belgium). The structures of the two drugs and their corresponding internal standards are shown in Fig. 1. HPLC grade methanol and acetonitrile were obtained from Lab Scan Analytical Services (Dublin, Ireland). Orthophosphoric acid AR and potassium dihydrogen orthophosphate AR were obtained from BDH (Poole, England). Filtration of HPLC mobile phases was performed using Gelman FP-450 filters (Pall Gelman Sciences, Northampton, England). Screened whole blood, from which drug free plasma was obtained, was kindly donated to the study by The Northern Ireland Blood Transfusion Service. Plasma was stored at −20 °C until required.

2.2. Instrumentation

The HPLC system employed in this study consisted of a Shimadzu SCL-10AVP system controller, a Shimadzu SIL-10ADVP autoinjector, a Shimadzu LC-10ATVP pump, a Shimadzu FCV-10ALVP solvent mixer, a Shimadzu DGU-14A degasser, a Shimadzu SPD-10AVP UV detector, a Shimadzu RF-10AXL fluorescence detector and a Jones Chromatography model 7990 column heater. Data were acquired and integrated using Shimadzu ClassVP software.

2.3. Ranitidine assay chromatographic conditions

Chromatographic separation was achieved using reverse-phase HPLC with isocratic elution. The method employed [3] was a modification of that described by López-Calull et al. [9] using AH20480 as the internal standard. The analytical column was a Waters μBondapak C18 (300 mm × 3.9 mm, 10 μm) fitted with a Waters μBondapak C18 (3.9 mm × 20 mm, 10 μm) guard column. UV detection at 313 nm was employed and the column temperature was maintained at 25 °C. An injection volume of 40 μL was utilised. The mobile phase consisted of acetonitrile:phosphate buffer (10 mM, pH 3.75) (13:87, v/v) filtered and degassed through a 0.45 μm filter. The mobile phase was delivered at a flow-rate of 1 mL/min. Peak area ratios were plotted against ranitidine concentration in the construction of the calibration curve.

2.4. Cisapride assay chromatographic conditions

Chromatographic separation was achieved using reverse-phase HPLC with isocratic elution. The system employed was a modification of that described by Preechagoon and Charles [1] using R-54680 as the internal standard. The analytical column was a Waters Symmetry® C_8 (150 mm × 3.9 mm, 5 μm) fitted with a Waters Symmetry® C_8 (3.9 mm × 20 mm, 5 μm) guard column. Fluorescence detection at an excitation wavelength of 295 nm and an emission wavelength of 350 nm was employed and the

column temperature was maintained at 25 °C. An injection volume of 20 μL was utilised. The mobile phase consisted of acetonitrile:phosphate buffer (0.02 M, pH 5.2) (370:630, v/v) filtered and degassed through a 0.45 μm filter. The mobile phase was delivered at a flow-rate of 1 mL/min. Peak area ratios were plotted against cisapride concentration in the construction of the calibration curve.

2.5. Collection of patient plasma samples

Patient blood samples were collected in 5 mL lithium-heparin sample tubes. The sample in the tube was inverted several times to ensure mixing and prevention of coagulation. The blood sample was then centrifuged for 10 min at $3000 \times g$ to separate the plasma component. Sample plasma was transferred to a clean sample tube and was stored at −20 °C prior to analysis.

2.6. Construction of a standard curve for ranitidine in plasma

Master stock solutions of ranitidine and AH20480 were prepared in methanol at a concentration of 1 mg/mL and were stored in the dark at 4 °C until required. Aqueous working standard solutions of ranitidine (10 μg/mL) and AH20480 (5 μg/mL) were prepared from the master stock standards. Plasma standards were prepared from the working ranitidine solution at concentrations of 10, 20, 50, 80, 100, 200, 500, 1000 and 2000 ng/mL by serial dilution into drug-free plasma. These ranitidine-plasma standards were then extracted by the developed solid phase extraction procedure that follows, with the internal standard working solution being added as appropriate in the protocol.

2.7. Construction of a standard curve for cisapride in plasma

Master stock solutions of cisapride and R-54680 were prepared in methanol at a concentration of 1 mg/mL and were stored in the dark at −70 °C until required. Working standard solutions of cisapride (1 μg/mL) and R-54680 (1 μg/mL) were prepared in methanol from the master stock standards and were stored in the dark at −70 °C until required. Plasma standards were prepared from the working cisapride solution at concentrations of 10, 20, 50, 100, 150, 250 ng/mL by serial dilution into drug-free plasma. These cisapride-plasma standards were then extracted by the solid phase extraction procedure that follows, with the internal standard working solution being added as appropriate in the protocol.

2.8. Development of an automated solid phase extraction procedure

The simultaneous separation of ranitidine and cisapride by solid phase extraction using disposable Oasis HLB cartridges (1 mL/30 mg, 30 mm; Waters, UK) was investigated by means of the following tabulated procedure [Table 1]. Minor modifications to this procedure are discussed in the text.

Table 1
Tabulated procedure for solid phase extraction development

Step	Source	Output	Volume (mL)	mL/min
Condition	MeOH	Waste 1	1	1
Condition	Water	Waste 1	1	1
Load	Sample	Waste 2	1.5	1
Rinse	5% methanol	Fraction 1	1	1
Rinse	20% methanol	Fraction 2	1	1
Rinse	40% methanol	Fraction 3	1	1
Rinse	60% methanol	Fraction 4	1	1
Rinse	80% methanol	Fraction 5	1	1
Rinse	90% methanol	Fraction 6	1	1
Rinse	MeOH	Fraction 7	1	1
Rinse	MeOH	Waste 1	2	1
Purge cannula	Water	Cannula	6	42
Purge cannula	MeOH	Cannula	6	42
Purge cannula	Water	Cannula	6	42

2.9. Solid phase extraction protocol

A simple one-dimensional SPE protocol was developed for the simultaneous extraction of ranitidine and cisapride from plasma using disposable Oasis HLB cartridges (1 mL/30 mg, 30 mm; Waters, UK). The procedure was carried out using a Zymark RapidTraceTM SPE Workstation. The plasma sample (calibrant or patient, 200 μL) was transferred to a disposable borosilicate glass culture tube and alkalinised with 1 M NaOH (140 μL), diluted to 1 mL by addition of 660 μL of HPLC grade water. Finally 10 μL of 10 mg/mL aqueous AH20480 and 20 μL of 1 mg/mL R-54680 were added to the sample. The plasma sample was placed in the appropriate position the sample rack. The sample rack was then loaded into the SPE Workstation.

Each cartridge was conditioned with methanol (1 mL) and water (1 mL). The plasma sample (1.03 mL—including both internal standards) was then loaded onto the cartridge. Sample interferents were washed from the cartridge with 40% aqueous methanol (1 mL). Cartridge-retained ranitidine and internal standard were then eluted with 70% aqueous methanol (1 mL). Cartridge-retained cisapride and internal standard were then eluted with 100% methanol (1 mL). Collected samples were eluted into disposable borosilicate glass culture tubes placed in the appropriate positions of the sample rack. Collected eluents were evaporated to dryness at 40 °C under a stream of N_2 and residues were reconstituted in 200 μL of the appropriate mobile phase before injection onto the HPLC system.

2.10. Recovery studies

The efficiency of the developed SPE extraction system was assessed by spiking drug-free plasma with known amounts of ranitidine (20, 100 and 750 ng/mL) and cisapride

(20, 100 and 250 ng/mL) and subsequently measuring the amount of spiked ranitidine and cisapride "recovered" following HPLC analysis of these samples. A calibration curve for ranitidine in mobile phase (incorporating AH20480 internal standard) and a calibration curve for cisapride in mobile phase (incorporating R-54680 internal standard) were constructed for this purpose. A ranitidine-free plasma sample and a cisapride-free plasma sample were also extracted and examined by HPLC to certify that no endogenous material interfered with either assay. In addition, an aqueous sample containing no ranitidine and an aqueous sample containing no cisapride were extracted using the SPE procedure to ensure no interferences from cartridge material.

2.11. Linearity, accuracy and sensitivity of HPLC methods for the determination of ranitidine and cisapride in plasma samples

The linearity of the developed ranitidine HPLC methods, for the determination of plasma-ranitidine concentrations, was assessed over a concentration range of 10–2000 ng/mL. Plasma-ranitidine standards (10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL) were prepared using drug-free plasma and working solutions of ranitidine and internal standard as previously described. The linearity of the developed cisapride HPLC methods, for the determination of plasma-cisapride concentrations, was assessed over a concentration range of 10–250 ng/mL. Plasma-cisapride standards (10, 20, 50, 100, 150, 250 ng/mL) were prepared using drug-free plasma and working solutions of cisapride and internal standard as previously described.

Each extracted standard was injected in triplicate, peak area ratios were calculated and the corresponding calibration curve was constructed accordingly. In this study, the accuracy of each developed HPLC methods was determined by measuring system precision and system trueness. The precision of both HPLC methods used in this study was evaluated by determination of intra- and interday percentage coefficients of variation (%CV values). Intraday variation was established on a single day by quintuplet estimation of drug-free plasma standards spiked with ranitidine or cisapride at three different concentrations 20, 750, 2000 ng/mL ranitidine; 20, 100, 250 ng/mL cisapride. Interday variation for both HPLC methods was established over a period of five days using the same methodology. The trueness of the HPLC methods used in the present study are expressed in terms of percentage bias, bias being defined as the positive or negative deviation of the mean analytical result from the known (or assumed) true value (EURACHEM 1998) [22]. Limits of quantitation (LOQ) and detection (LOD) were used to evaluate the sensitivity of the developed HPLC methods for the determination of plasma-ranitidine or plasma-cisapride concentrations. In this study, the LOQ was defined as the lowest concentration point on the ranitidine-plasma calibration curve (EURACHEM 1998; FDA 1998) [22,23]. The limit of detection (LOD) was defined as the concentration of ranitidine in plasma that would provide a signal equivalent to three times the signal to noise level (SN).

3. Discussion

Initial attempts at developing a single method for the analysis of combinations of ranitidine and cisapride in biological matrices involved investigations of the HPLC methods previously used, by us, for the determination of the individual drugs. The ranitidine assay utilised a reversed phase system with UV detection whilst the cisapride assay involved a different reversed phase system and fluorescence detection. In these initial investigations we used chromatographic conditions as for the individual assays with a UV and a fluorescence detector in series. Using the cisapride HPLC method we employed a Waters Symmetry® C_8 (150 mm × 3.9 mm, 5 μm) column and a mobile phase of 63/37 phosphate buffer (0.02 M, pH 5.2): AcCN. In this system ranitidine and the internal standard [IS] (AH20480) eluted immediately following the solvent and where the endogenous components of the plasma eluted. Modification of the mobile phase from 63/37 phosphate buffer: AcCN to 70:30 resulted in a small increase in the retention of ranitidine and IS and a substantial increase (more than two-fold) in the retention of cisapride and its internal standard (R-54680). Further changes in the mobile phase composition from 70/30 phosphate buffer: AcCN to 80:20 and subsequently 90:10 again resulted in a small increases in the retention of ranitidine and IS although these two components still eluted with the plasma components. Under these conditions the cisapride and its IS were not eluted from the column within 60 min.

The HPLC method [15] that had been used successfully for the analysis of ranitidine plasma samples was then investigated. With this system [mobile phase 87:13 phosphate buffer (10 mM, pH3.75):AcCN] retention times of 6.6 and 10.3 min were obtained for ranitidine and AH20480, respectively. Following injection of samples of cisapride and its internal standard R-54680 onto this system no peaks were observed for either compound even after 30 min. With the mobile phase adjusted to 75:25 neither of the aforementioned compounds eluted within 30 min. When the mobile phase was changed to 60:40 phosphate buffer:AcCN cisapride eluted at approximately 10 min and the IS at 15 min, however, the ranitidine eluted with the solvent front.

The possible utilisation of gradient elution systems based on the previous investigations of methods for the individual drugs was then investigated, however this proved unsuccessful. As an example, when the cisapride system was utilised with a linear gradient from 90:10 to 63:37 (phosphate buffer:AcCN) over a period of 30 min, cisapride and R-54680 eluted at 18 and 22 min respectively whilst ranitidine and AH20480 eluted with the solvent front. Adjustment of this linear gradient from 90:10 to 70:30 (phosphate buffer:AcCN) over a period of 30 min led to an increase in the retention times of cisapride (RT = 22 min) and R-54680

(27 min) but ranitidine and AH20480 still eluted with the solvent front. Also the baseline from the fluorescence detector (Preechagoon and Charles have utilised fluorescence detection for its enhanced sensitivity and selectivity for cisapride determination) [19] was considerably affected by the gradient such that the sensitivity for accurate quantification at the levels required for cisapride would have been impossible.

It was concluded that the development of a single, routine HPLC procedure for the determination of cisapride and ranitidine (along with their internal standards) was unlikely due to the significant differences in the physicochemical characteristics of these two drugs. The log *P* [octanol/water] for cisapride and ranitidine are, respectively, 3.96 and 0.27 [24].

Because of these differing physical characteristics it was decided to investigate the possibility of separating cisapride from ranitidine by means of solid phase extraction using OASIS^{TM} HLB cartridges. Initial studies involved investigation of the elution profile of the individual drugs from the cartridges. The cartridges were firstly conditioned using methanol and water according to normal procedures. The sample [drug plus appropriate IS] was loaded onto the cartridge and the cartridge was then rinsed with methanol/water mixtures in which the concentration of methanol was gradually increased (5, 20, 40, 60, 80, 90, 100%) see Table 1. Each fraction was collected and analysed using the appropriate HPLC method. From these investigations it was observed that neither compound was detected in the collected load sample fraction or the conventional 5% methanol wash phase. The ranitidne and internal standard were completely eluted from the cartridges in the fractions containing 60 and 80% methanol whilst cisapride and IS only eluted in the 100% methanol fraction. Thus, it appeared that the HLB solid phase extraction material was capable of separating the ranitidine and cisapride (and their respective internal standards) "on cartridge". This was confirmed following further investigations using combined samples of the drugs (plus IS) and studies involving spiked plasma samples.

Subsequently a method was developed for the analysis of these components in paediatric plasma samples, this method is described in Section 2 [solid phase extraction protocol]. In this method, sample clean up (in which plasma interferents were washed from the column using 40% methanol) was carried out before elution of ranitidine and internal standard in the 70% methanol fraction. Cisapride and IS were only eluted in the 100% methanol fraction.

Having developed a SPE method for the separation of the two study drugs (and their internal standards) we then proceeded to develop and validate a method for the determination of both drugs in paediatric plasma samples. The assay procedure for ranitidine has recently been reported by our group [15] the assay being linear ($r^2 = 0.9997$) over the range 8–800 ng/mL with a LOQ of 8 ng/mL and a LOD of 2 ng/mL. The determination of cisapride was carried out using the method of Prechagoon and Charles [19] that had been specifically developed for the assay of cisapride in neonatal plasma. Fig. 2 shows representative chromatograms following the extraction of blank plasma, the chromatograms show the 70% methanol fraction where ranitidine (plus IS) (a) is eluted and the 100% methanol fraction where cisapride and its IS elute (b). Fig. 3 represents chromatograms

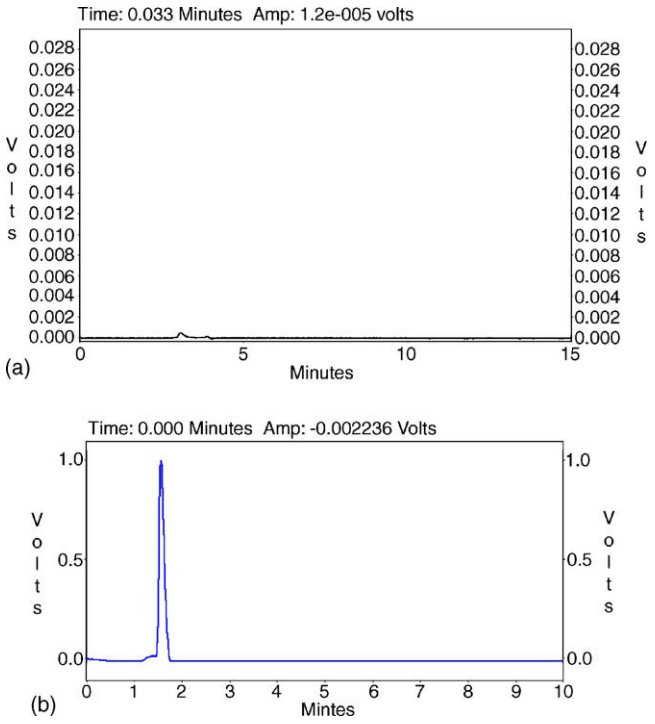

Fig. 2. Representative chromatograms of the 70% methanol and 100% methanol fractions from the SPE of a blank plasma sample.

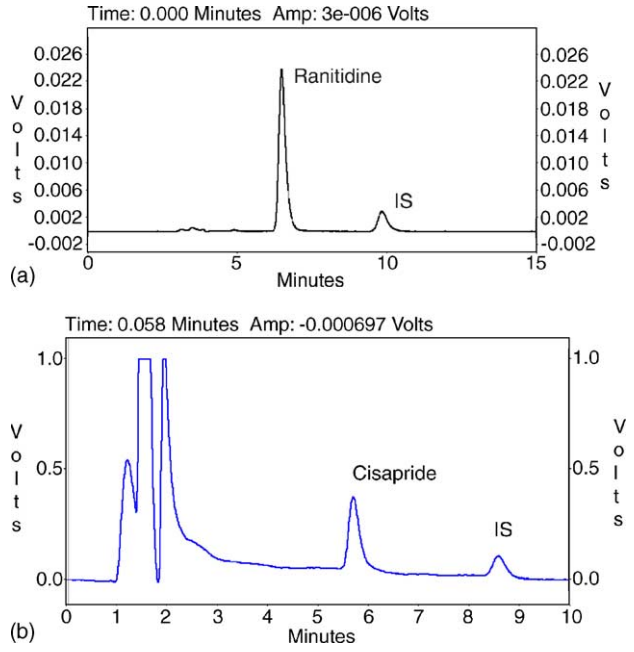

Fig. 3. Representative chromatograms illustrating the collected fractions containing ranitidine (a) and cisapride (b) following SPE of spiked plasma samples.

Table 2
Recovery of ranitidine from spiked drug-free plasma following solid phase extraction

Concentration ranitidine spike (ng/mL)	% Recovery	% CV	*n*
20	86.6	6.4	5
750	90.6	11.4	5
2000	97.0	2.6	5

Table 3
Recovery of cisapride from spiked drug-free plasma following solid phase extraction

Concentration cisapride spike (ng/mL)	% Recovery	% CV	*n*
20	85.2	0.6	5
100	102.9	7.8	5
250	100.8	7.1	5

of spiked plasma obtained following the use of the developed SPE methodology for ranitidine (a) and cisapride (b), respectively.

Recovery experiments were conducted using drug free plasma spiked with cisapride and ranitidine. The results are presented in Tables 2 and 3 and show that in all cases the recoveries were in excess of 85%. The recoveries reported are slightly lower than those we have achieved in the developed methods for the quantification of the drugs individually. Data for precision and bias for both cisapride and ranitidine are shown in Tables 4 and 5. Whilst this is not an ideal method in that two separate chromatographic methods had to be employed for the analysis of the two drugs, the method did enable us to determine the drug concentrations in those patients taking both drugs simultaneously. The possible use of column switching techniques incorporating Oasis HLB cartridge columns might prove a useful alternative in such studies although this option was not available in our laboratory at the time of this study.

This methodology has been successfully applied to the analysis of 60 paediatric plasma samples containing both cisapride and ranitidine (see Fig. 4). The quantitative data obtained is now being investigated (along with data from paediatric patients receiving either drug individually) by means of sparse data analysis.

We have also shown that other compound mixtures with significantly different log *P* values can be separated using these SPE cartridges, e.g. spironolactone and frusemide (log *P* 2.260 and −1.060, respectively) [24]. Further work is underway to investigate the utilisation of such "on-cartridge" separations for the analysis of co-formulated drug products.

Table 4
Precision and bias of HPLC method for the quantification of ranitidine in plasma

Concentration ranitidine added to combination plasma sample (ng/mL)	Concentration ranitidine quantified (ng/mL)	% CV	% Bias	*n*
Intra-day				
20	20.6	7.7	+2.8	5
750	659	3.0	−12.2	5
2000	1760	0.9	−12.2	5
Inter-day				
20	18.3	12.3	−8.3	5
750	744	11.4	−0.8	5
2000	1960	7.6	−2.2	5

Table 5
Precision and bias of HPLC method for the quantification of cisapride in plasma

Concentration cisapride added to combination plasma sample (ng/mL)	Concentration cisapride quantified (ng/mL)	% CV	% bias	*n*
Intra-day				
20	20.5	14.8	+2.4	5
100	88.7	1.5	−11.3	5
250	261	3.6	+4.5	5
Inter-day				
20	20.7	21.8	+3.4	5
100	99.7	8.8	−0.3	5
250	256	4.5	+2.3	5

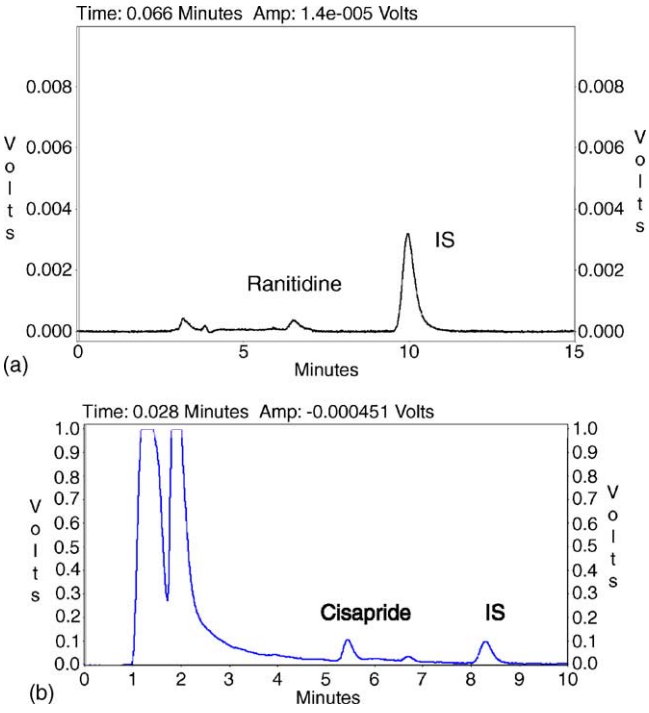

Fig. 4. Representative chromatograms illustrating the collected fractions containing ranitidine (a) and cisapride (b) following SPE of patient plasma samples.

Acknowledgements

The financial assistance of Action Research (grant S/P/3273) is gratefully acknowledged.

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