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Simultaneous determination of paracetamol and chlorpheniramine in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

An analytical method for the determination of paracetamol and chlorpheniramine in human plasma has been developed, validated and applied to the analysis of samples from a phase I clinical trial. The analytical method consists in the extraction of paracetamol and chlorpheniramine with diethyl ether, followed by the determination of both drugs by an LC–MS–MS method, using 2-acetamidophenol as internal standard. The intra-assay and inter-assay precision and accuracy of this technique were good and the limit of quantitation was 0.5 μg/ml of plasma for paracetamol and 0.2 ng/ml for chlorpheniramine. The concentration working range was established between 0.5 μg/ml and 25 μg/ml for paracetamol and between 0.2 ng/ml and 50 ng/ml for chlorpheniramine. This method has been used for analyzing more than 1200 human plasma samples from a clinical study with 24 volunteers.

Keywords: Validation; Paracetamol; Chlorpheniramine

1. Introduction

Paracetamol (acetaminophen, *N*-acetyl-*p*-aminophenol) is a safe and effective analgesic and antipyretic agent although its anti-inflammatory effect is weak. In case of an overdose, it may cause severe hepatic necrosis [1,2]. Paracetamol is rapidly absorbed from the gastrointestinal tract but it is incompletely available due to first pass effect [3–5]. Extensive metabolism occurs, predominantly in the liver, the major metabolites being the sulfate and the glucuronide conjugates [6]. Several methods, many of them involving reversed-phase liquid chromatography (LC) with UV detection, have been proposed for the determination of paracetamol in biological fluids [7–9].

Chlorpheniramine [3-(*p*-chlorophenyl)-3-(2-pyridyl)-*N*,*N*-dimethylpropylamine] is a powerful H_1-receptor antagonist (anti-histamine), widely used for symptomatic relief of common cold and allergic rhinitis, with weak sedative properties [10].

Despite its widespread use over an extended period, relatively low, and in many cases contradictory pharmacokinetic data are available for chlorpheniramine. Several analytical methods including high-performance liquid chromatography (HPLC) with UV [11,12] or fluorescence [13] detection, radioimmunoassay [14] or gas–liquid chromatography [15,16] have been employed but, as a major drawback, many of these methods require derivatization and/or several extraction steps, which makes them tedious and time consuming.

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In this paper we describe a method to simplify, to speed-up and to assay simultaneously paracetamol and chlorpheniramine in plasma using the LC–tandem mass spectrometry (MS–MS) technique. Both drugs are extracted with diethyl ether and analyzed by LC–MS–MS. This method has been used to compare paracetamol and chlorpheniramine plasma levels in a clinical study with 24 healthy volunteers after administration of single oral doses in three different formulations, one association of *N*-acetylcysteine (200 mg), paracetamol (650 mg) and chlorpheniramine (4 mg), a second formulation with only paracetamol (650 mg) and a third formulation with only chlorpheniramine (4 mg).

2. Experimental

2.1. Materials and reagents

Paracetamol (99.0%) and chlorpheniramine maleate (>99%) were from Sigma (Steinheim, Germany). 2-Acetamidophenol (97%) was obtained from Aldrich (Steinheim, Germany). Acetonitrile and diethyl ether (HPLC-grade), sodium hydroxide (analytical-reagent grade), formic acid (98–100%), boric acid (analytical-reagent grade) and potassium chloride (analytical-reagent grade) were from Merck (Darmstadt, Germany). Pentafluoropropionic anhydride (>98%) was obtained from Lancaster (Morecambe, UK). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA) until a resistivity of 18 MΩ cm was achieved.

KCl–H_3BO_3–NaOH, pH 8.0 buffer was prepared as follows: 37.28 g of KCl and 30.92 g of H_3BO_3 were dissolved in 1 l of water and 50 ml of this solution was mixed with 3.9 ml of 0.5 *M* NaOH and diluted to 100 ml in a volumetric flask. The final pH of the solution was checked using a pH meter.

Heparinized human plasma was obtained from untreated healthy volunteers (0.75 mg sodium heparinate/ml of blood, using an aqueous 5% sodium heparinate solution). Plasma was obtained by centrifugation of blood at 2000 *g* for 15 min at 4°C. Plasma obtained from each volunteer was pooled and frozen at −20°C to be used during the study.

2.2. Preparation of calibration curves and quality controls

Standard stock solutions of paracetamol and chlorpheniramine (1 mg/ml) in water–acetonitrile (80:20) were prepared separately. After an appropriate dilution procedure in the same solvent, an equal volume of each final solution was mixed and a serial dilutions scheme in the same solvent was applied to get the suitable range of calibration standard solutions of 500, 250, 150, 100, 50, 10, 5 and 1 μg/ml for paracetamol and 1000, 500, 300, 200, 100, 20, 10 and 2 ng/ml for chlorpheniramine. An internal standard solution of 10 μg/ml in the same solvent was also prepared.

Calibration curves were prepared by spiking 50 μl of the appropriate standard solution to 0.5 ml of untreated human plasma. Effective concentrations in plasma samples were 25, 15, 10, 5, 1, 0.5 and 0.1 μg/ml for paracetamol, and 50, 30, 20, 10, 2, 1 and 0.2 ng/ml for chlorpheniramine. One plasma blank with 100 μl of water–acetonitrile (80:20) was added to each calibration curve. The quality controls (QCs) used in the validation were prepared in the same way as the calibration standards, its nominal concentrations being 0.10, 0.50, 5 and 15 μg/ml for paracetamol and 0.2, 1, 10 and 30 ng/ml for chlorpheniramine. For real sample analysis, quality controls nominal plasma concentrations of paracetamol were 0.5, 5 and 15 μg/ml and 1, 10 and 30 ng/ml for chlorpheniramine.

2.3. Sample preparation

Calibration curves, quality controls and real samples were treated as follows: 0.5 ml of KCl–H_3BO_3–NaOH, pH 8 buffer solution were added to each plasma sample after being spiked with 50 μl of working internal standard solution, except for the blanks. An extraction with 2 ml of diethyl ether was carried out for 10 min using a tube shaker. The tubes were then centrifuged at 2000 *g* for 15 min and the organic layer separated by means of an acetone–dry ice bath, and then transferred to microcentrifuge polypropylene tubes for evaporation in a rotational vacuum concentrator (Alpha-RVC Christ, Osterode am Harz, Germany), initially at 30°C and 500 mbar for 35 min and, in a second cycle, at 25 mbar for 5

min keeping the same temperature. The residue was resuspended in 250 μl of water–acetonitrile (80:20). The resuspended sample was transferred to a clean low-volume autosampler vial and 50 μl was injected into the LC–MS–MS system.

2.4. Method validation

Weighed least-squares (weighing factor= concentration^{-2}) was used to fit the response of the data versus the effective concentration to the equation: area response=slope×concentration±intercept for chlorpheniramine. In the case of paracetamol, least-squares was used to fit response of data versus the effective concentration to the equation: area response=A(concentration)^2+B(concentration)+C. The backcalculated values of the concentrations were statistically evaluated.

The intra-assay precision and accuracy were determined by analyzing a set of QC samples (n=6) at each of the four levels, 0.1, 0.5, 5 and 10 μg/ml for paracetamol and 0.2, 1, 10 and 30 ng/ml for chlorpheniramine using calibration curves obtained from duplicate standards. The inter-assay precision and accuracy studies were also carried out by analyzing QC samples (n=2) at the above four concentrations for each drug a further three times.

2.5. Recovery

Absolute recoveries for paracetamol (0.5, 5 and 15 μg/ml), chlorpheniramine (1, 10 and 30 ng/ml) and the internal standard (1000 ng/ml) from human plasma were calculated by comparing the area ratios of the plasma samples with reference samples. Reference samples were prepared by addition to blank plasma extracts 50 μl of the suitable calibration standard solution and the adequate volume of acetonitrile and water to obtain the same final concentration of drugs as the replicate plasma samples, that were prepared as the calibration standards; the number of replicates for each concentration was six.

2.6. Stability

Stability of paracetamol and chlorpheniramine under the experimental conditions was investigated by comparison of the mean area ratios obtained in each case with the mean areas from a control set of replicates (time zero). The concentration of the spiked plasma samples was 1 and 15 μg/ml for paracetamol and 2 and 30 ng/ml for chlorpheniramine. The number of replicates used for each determination was five. Results were expressed for each compound and concentration level as the percentage of the initial control concentration (C_0) which is referred to as 100%. Stability was studied for the dry residue after 24 and 72 h at 4°C, under autosampler conditions after 24 h at room temperature, for up to three freeze–thaw cycles and for 1 and 3 months stored below −20°C.

2.7. LC–MS–MS

LC was performed using two Applied Biosystems (San Jose, CA, USA) SF-400 pumps and a Gilson 233XL (Villiers-le-Bel, France) autosampler. The LC system was coupled to an API 365 PE-Sciex (Concord, Canada) turboionspray tandem mass spectrometer. The separation was achieved on a 5 μm Kromasil C_{18} column (50×4.6 mm I.D.). Elution was achieved at room temperature with water–acetonitrile (80:20), 0.5% formic acid and 1 mM pentafluoropropionic anhydride as the mobile phase. The LC system was operated isocratically at 1 ml/min. The column eluent was split and approximately 200 μl/min was introduced in the mass spectrometer. Turboionspray temperature was set at 350°C, using nitrogen (5.0 grade) at 7 l/min as auxiliary gas and zero grade air as nebulizer gas at a pressure of 90 p.s.i. (1 p.s.i.=6894.76 Pa). The ionspray voltage was set at 4000 V and the orifice voltage was set at 5 V. The instrument was programmed for a scan dwell time of 500 ms. The responses of paracetamol, chlorpheniramine and the internal standard were measured in the positive ion mode using the MRM (multiple reaction monitoring) technique. The protonated molecular ions of paracetamol and the internal standard 2-acetamidophenol (m/z 152) as well as the chlorpheniramine (m/z 275) were dissociated by collision with nitrogen at a gas thickness of $1.56 \cdot 10^{15}$ molecules cm^2 and under an energy of 21 eV. The mass spectrometer was set to selectively monitor parent to daughter fragments of m/z 152→110 for paracetamol and 2-acetamidophenol

and m/z 275→230 for chlorpheniramine. Data were acquired and analyzed using the MassChrom 1.0 software package, running on a Power Macintosh 7300/200 computer.

3. Results and discussion

3.1. Mass spectra

LC–MS–MS for the determination of paracetamol chlorpheniramine and 2-acetamidophenol in human plasma was investigated. Positive electrospray mass spectra of paracetamol and 2-acetamidophenol show an intense $[M+H]^+$ ion at m/z 152. Another intense $[M+H]^+$ ion at m/z 275 is shown for chlorpheniramine. When these molecular ions undergo fragmentation in the collision cell, the product ion mass spectra shown in Fig. 1 are generated. With the experimental conditions used in these experiments, paracetamol and 2-acetamidophenol show an intense product ion at m/z 110 which corresponds to the ion $^+NH_3$-C_6H_4-OH obtained after a $CO{=}CH_2$ neutral loss (Fig. 1a); (2-acetamidophenol shows the same spectrum than paracetamol). The product ion mass spectra of chlorpheniramine shows an intense fragment at m/z 230 that would correspond to an ionic fragment obtained after the loss of dimethylamine $[NH(CH_3)_2]$ (Fig. 1b).

3.2. Separation and specificity

Three typical MRM chromatograms from the study of paracetamol and chlorpheniramine in human plasma are shown in Fig. 2. Retention times of less than 3 min were achieved for paracetamol and the internal standard that are completely separated and less than 4 min for chlorpheniramine. No interference peaks were found in the MRM profiles for six blank plasma samples (see Fig. 2a). MRM chromatograms of blank plasma spiked with known amounts of paracetamol, chlorpheniramine and 2-acetaminophenol are shown in Fig. 2b and a sample from a volunteer is shown in Fig. 2c. For both drugs and the internal standard, the chromatograms were free of interfering peaks at their respective retention times.

3.3. Linearity

The assay followed a quadratic equation for paracetamol in the concentration range of 0.5–25 μg/ml. The standard of concentration of 0.1 μg/ml had to be included in the calculations of the curve to produce a good fit of the 0.5 μg/ml point. In some way, the standard of 0.1 μg/ml is doing the same work as the weight factor used in the linear regression analysis, which in the software used was not available for quadratic equation. Precision of the back-calculated concentrations was between 0.23 and 5.11% and the relative error (RE) was in the range of −6.58–6.07%. For chlorpheniramine, the assay was linear in the concentration range of 0.2–50 ng/ml. Precision of the back-calculated concentrations was between 1.44% and 8.57% and the relative error was in the range of −3.17–5.09%. Correlation coefficients (r) were between 0.9955 and 1.0000.

3.4. Accuracy and precision

Table 1 shows the accuracy and precision intra- and inter-assay for paracetamol. Relative standard deviations (RSDs) were in the range of 4.60–10.06% and REs were between −10.93 and 11.24%. The limit of quantitation was established as 0.5 μg/ml, its accuracy and precision values being −10.93% and 10.06%, respectively.

Table 2 shows the accuracy and precision intra- and inter-assay for chlorpheniramine. RSDs were in the range of 3.21–10.58% and REs were between −12.07 and 4.68%. The limit of quantitation was established as 0.2 ng/ml, its accuracy and precision values being −3.92% and 10.58%, respectively. These results indicate that the method was reliable within the analytical range, and the use of the internal standard was very effective for reproducibility by LC–MS–MS.

3.5. Recovery

Mean absolute recoveries for paracetamol at 0.5, 5 and 15 μg/ml were 17.7%, 20.1% and 23.6%, respectively. For chlorpheniramine the recovery values were 75.2%, 76.6% and 78.7% for 1, 10 and 30 ng/ml, respectively. Mean recovery for the internal standard (1000 ng/ml) was 47.3%. The low recovery

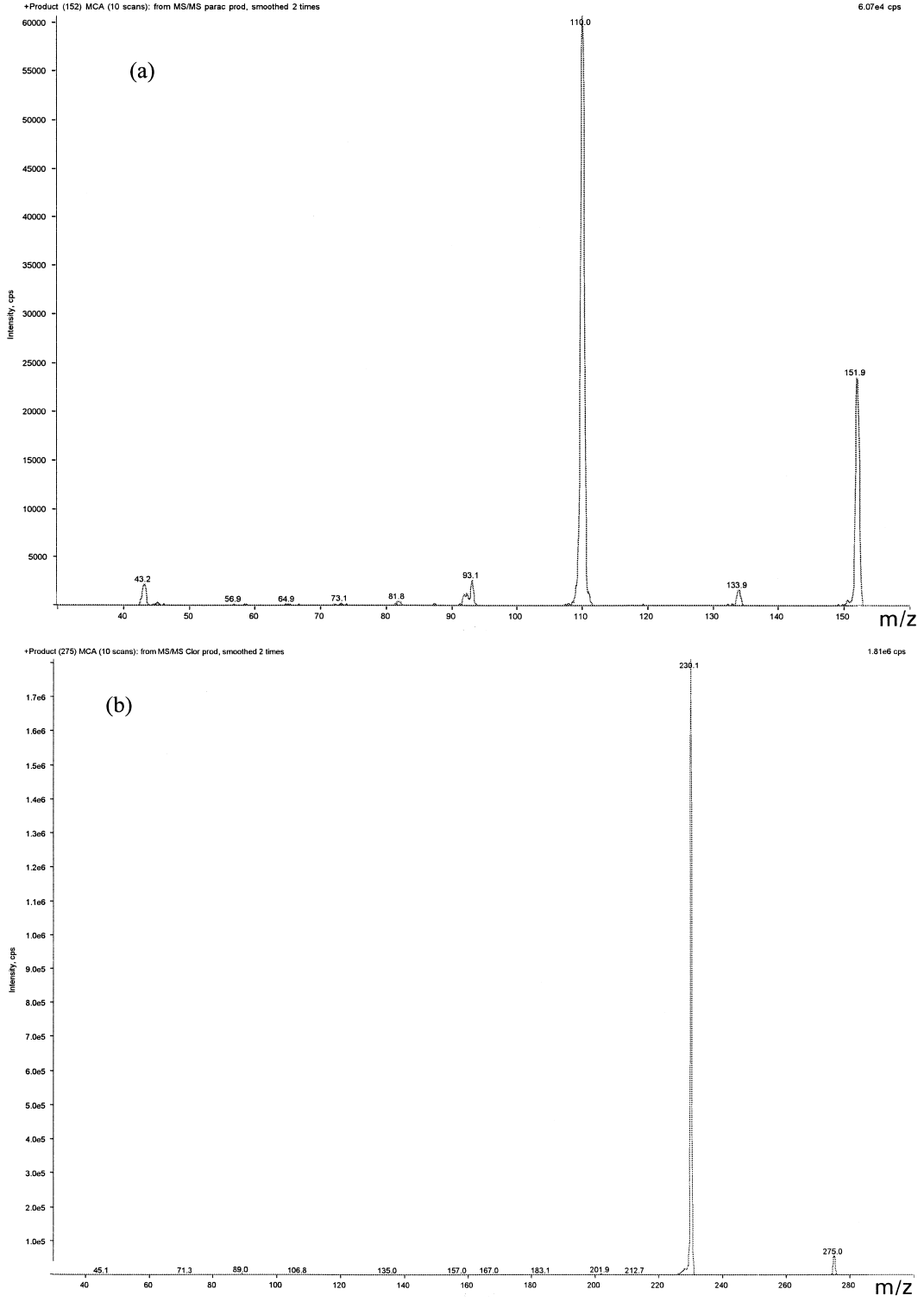


Fig. 1. Product ion mass spectra: (a) paracetamol and (b) chlorpheniramine.

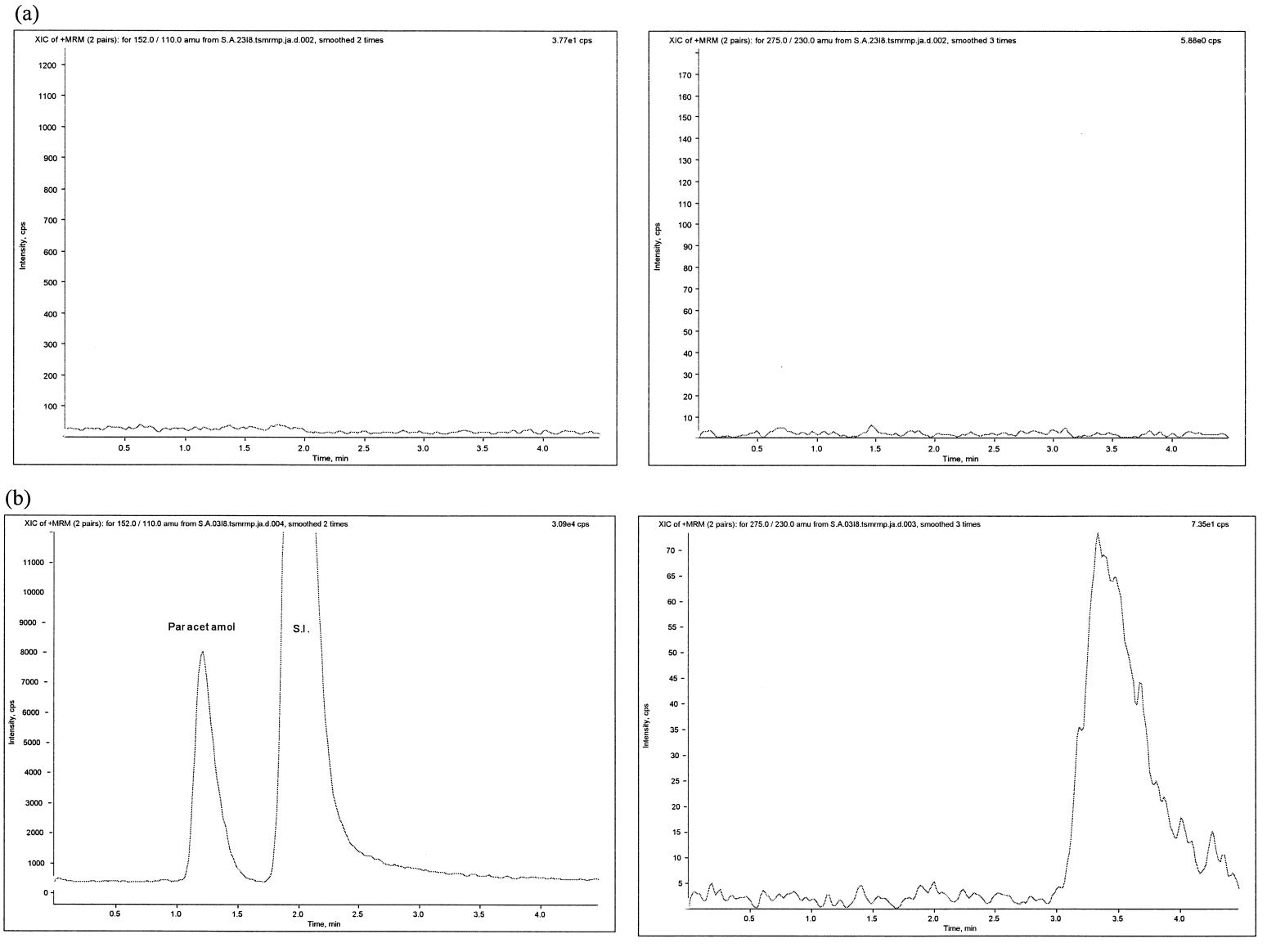
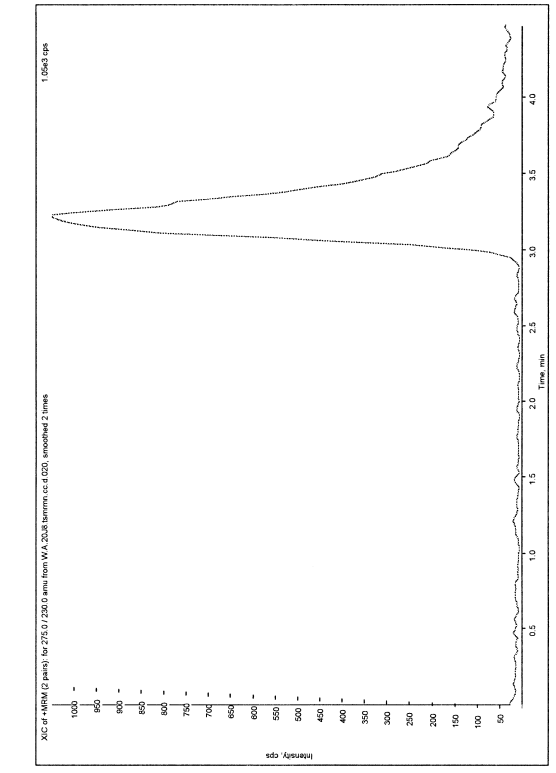


Fig. 2. Representative MRM chromatograms of paracetamol and 2-acetamidophenol (left) and chlorpheniramine (right): (a) blank human plasma, (b) human plasma spiked with paracetamol (0.5 μg/ml), chlorpheniramine (0.2 ng/ml) and internal standard (1 μg/ml) and (c) a sample from a volunteer (4.3 μg/ml of paracetamol and 3.9 ng/ml of chlorpheniramine) 1.5 h after oral administration of 650 mg of paracetamol and 4 mg of chlorpheniramine.



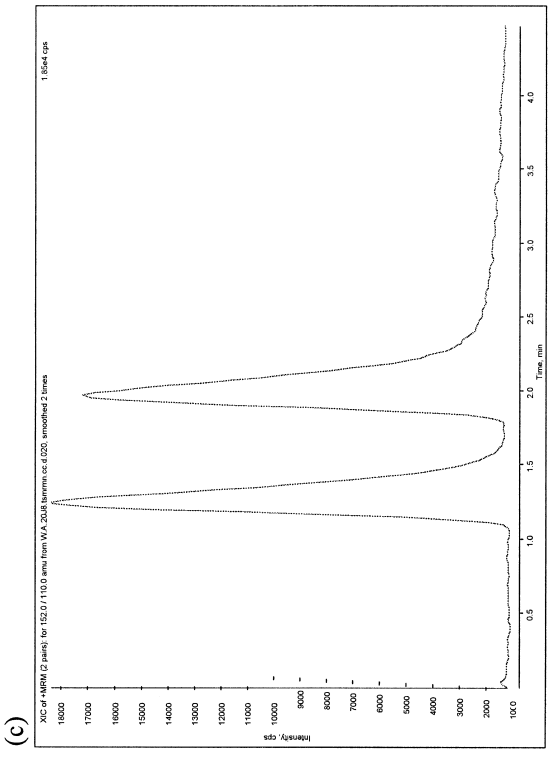


Fig. 2. (*continued*)

Table 1
Precision and accuracy for paracetamol

Effective concentration (μg/ml)	Mean calculated concentration (μg/ml)	SD	RSD (%)	RE (%)	*n*
Intra-assay					
0.5	0.45	0.04	9.82	−10.93	6
5	5.13	0.24	4.60	2.67	6
15	14.46	0.70	4.86	−3.61	6
Inter-assay					
0.5	0.47	0.05	10.06	−6.45	8
5	5.56	0.27	4.85	11.24	8
15	15.57	0.78	5.02	3.83	8

values found for paracetamol and the internal standard can be presumably due to the alkaline conditions of the extraction step considering that the phenol group of both compounds can be partially ionized under these conditions. Nevertheless, this low but constant recovery of paracetamol is not a drawback for the simultaneous analysis of both compounds. Due to the huge difference in the administered doses of each drug (more than 160-fold) and in the plasma levels found (μg range for paracetamol versus ng/ml for chlorpheniramine), it could be thought that a better recovery in paracetamol would produce an increase in the loss of linearity due to a saturation phenomena, that in fact may be happening now.

3.6. Stability

Resuspended plasma extracts of both paracetamol and chlorpheniramine were found to be stable for 24 h in the autosampler at room temperature. Also both drugs resist the effect of up to three freezing and thawing cycles and the storage in plasma below −20°C up to 3 months. Dry residue plasma extracts kept at 4°C showed contradictory results after 24 and 72 h in both paracetamol and chlorpheniramine, and we decided to resuspend the samples immediately after the evaporation step was done.

3.7. Application

This validated analytical method has been used in the determination of around 1200 plasma samples from a phase I clinical trial in which 24 healthy volunteers received 650 mg of paracetamol and 4 mg of chlorpheniramine. Fig. 3a shows a profile of the mean plasma concentrations ($n=24$) of paracetamol

Table 2
Precision and accuracy for chlorpheniramine

Effective concentration (ng/ml)	Mean calculated concentration (ng/ml)	SD	RSD (%)	RE (%)	*n*
Intra-assay					
0.2	0.19	0.02	10.58	−3.92	6
1	0.88	0.05	5.26	−12.07	6
10	9.90	0.32	3.21	−1.01	6
30	30.29	1.68	5.56	0.98	6
Inter-assay					
0.2	0.20	0.02	10.25	0.00	8
1	0.93	0.07	7.89	−6.96	8
10	10.04	0.44	4.41	0.45	8
30	31.40	2.30	7.31	4.68	8

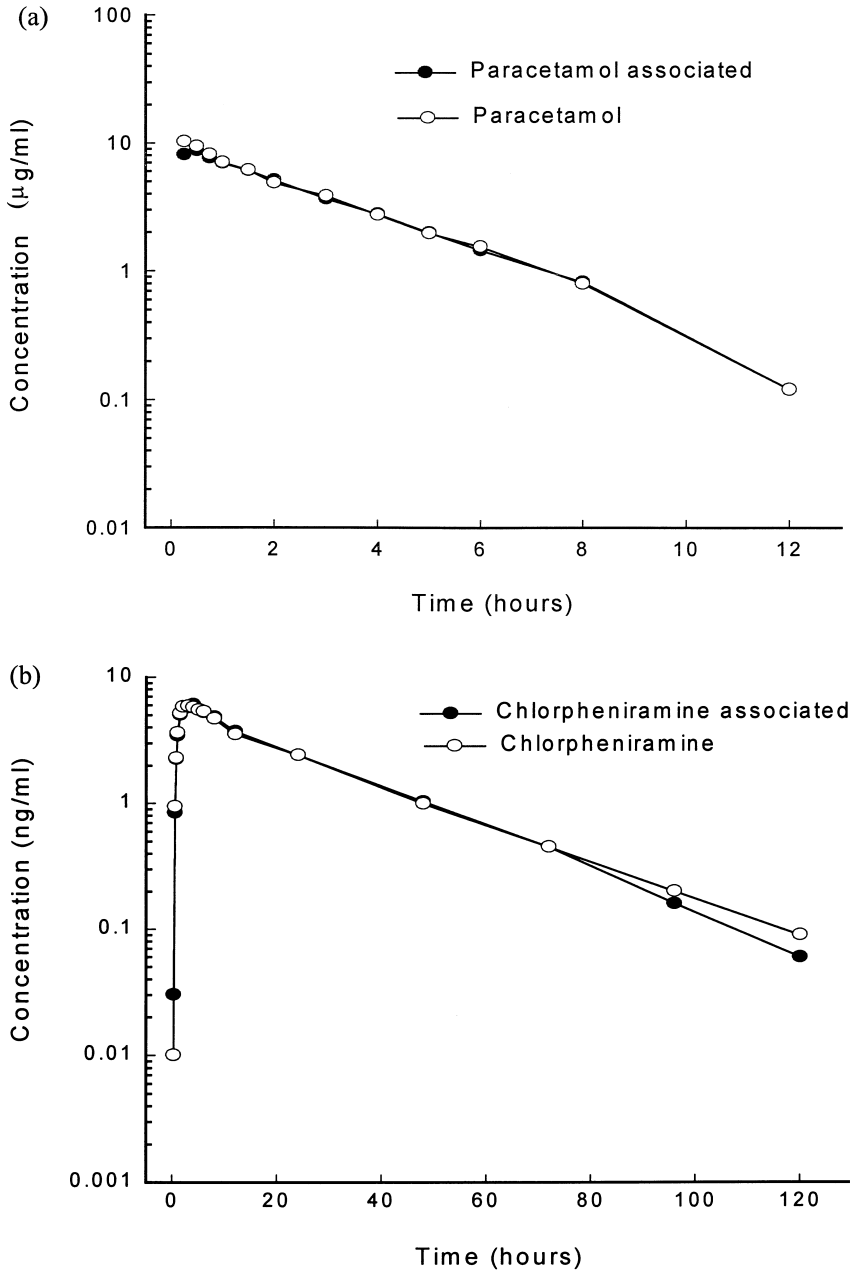


Fig. 3. Mean plasma concentrations (n=24) for two dosage forms: (a) paracetamol and (b) chlorpheniramine.

versus time for two formulations: one associated with *N*-acetylcysteine and chlorpheniramine and another one with paracetamol only. Fig. 3b shows a profile of the mean plasma concentrations (n=24) of chlorpheniramine versus time for two formulations: one associated with *N*-acetylcysteine and paracetamol and another one with chlorpheniramine only. The calculation of precision and accuracy of the quality controls used during the study (see Table 3) evaluated the quality of the analytical data.

Table 3
Quality control precision and accuracy for paracetamol and chlorpheniramine in the analysis of real samples

Effective concentration	Mean calculated concentration	SD	RSD (%)	RE (%)	*n*
Paracetamol (μg/ml)					
0.5	0.50	0.05	9.85	0.40	50
5	5.25	0.32	6.01	5.04	50
15	15.24	1.14	7.46	1.60	50
Chlorpheniramine (ng/ml)					
1	1.01	0.11	10.88	1.42	50
10	10.54	1.11	10.53	5.45	50
30	30.01	3.16	10.55	0.02	50

4. Conclusions

The use of LC–MS–MS allows for accurate, precise and reliable simultaneous measurement of paracetamol and chlorpheniramine concentrations in human plasma after oral administration of 650 mg and 4 mg, respectively, to healthy volunteers. The assay has proven to be fast and rugged, with each sample requiring less than 4.5 min of run time. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The method described here has significant advantages over the other techniques used for measuring both compounds in biological fluids. The major advantages of this method are the simple preparation and the rapidity of separation.

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