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Simultaneous determination of dextromethorphan, dextrorphan and doxylamine in human plasma by HPLC coupled to electrospray ionization tandem mass spectrometry: Application to a pharmacokinetic study

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ABSTRACT

In the present study, a fast, sensitive and robust method to quantify dextromethorphan, dextrorphan and doxylamine in human plasma using deuterated internal standards (IS) is described. The analytes and the IS were extracted from plasma by a liquid–liquid extraction (LLE) using diethyl-ether/hexane (80/20, v/v). Extracted samples were analyzed by high performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS). Chromatographic separation was performed by pumping the mobile phase (acetonitrile/water/formic acid (90/9/1, v/v/v) during 4.0 min at a flow-rate of 1.5 mL min^{-1} into a Phenomenex Gemini® C18, 5 μm analytical column (150 × 4.6 mm i.d.). The calibration curve was linear over the range from 0.2 to 200 ng mL^{-1} for dextromethorphan and doxylamine and 0.05 to 10 ng mL^{-1} for dextrorphan. The intra-batch precision and accuracy (%CV) of the method ranged from 2.5 to 9.5%, and 88.9 to 105.1%, respectively. Method inter-batch precision (%CV) and accuracy ranged from 6.7 to 10.3%, and 92.2 to 107.1%, respectively. The run-time was for 4 min. The analytical procedure herein described was used to assess the pharmacokinetics of dextromethorphan, dextrorphan and doxylamine in healthy volunteers after a single oral dose of a formulation containing 30 mg of dextromethorphan hydrobromide and 12.5 mg of doxylamine succinate. The method has high sensitivity, specificity and allows high throughput analysis required for a pharmacokinetic study.

1. Introduction

Dextromethorphan (DXM) is a drug that has been widely used for more than 40 years. Dextromethorphan (d-3-methoxymorphinan) is the dextro-isomer of levorphanol and an analog of codeine and a semisynthetic morphine derivative [1]. Dextromethorphan has no agonist activity at the opioid receptors, but it acts centrally to elevate the threshold for coughing [2]. At therapeutic doses, DXM produces minimal analgesic and antitussive effects and has been used for the relief of coughs caused by minor viral upper respiratory tract infections or inhaled irritants and is thought to be most effective for a chronic, but nonproductive cough. Dextromethorphan was approved by the US FDA in 1958 as an OTC cough suppressant and it has been excluded from the US Drug Enforcement Administration (DEA) Controlled Substance Act since 1970.

Like many of the H1-receptor antagonists (antihistamines), doxylamine succinate exhibits anti-emetic properties and is used therapeutically for the treatment of nausea and vomiting [3]. Doxylamine succinate is well absorbed after oral administration, extensively metabolized in the liver (mainly by hepatic microsomal oxidation) and reaches a peak plasma concentration within 2–3 h [4].

While there are several known metabolites of dextromethorphan [5,6], the parent drug and its unconjugated metabolite, dextrorphan, are the two molecules primarily responsible for antitussive activity [7–9]. Therefore, it is of interest to measure the plasma concentrations of both compounds to assess the relationship between concentration and antitussive response.

Several analytical approaches for measuring human plasma levels of dextromethorphan and/or dextrorphan have been reported in the literature. Most of these methods are based on chromatographic separations and utilize various detection schemes including LC-fluorescence [10–12], LC–UV [13], CE–UV [14], and GC–NPD [15]. In recent years, several liquid chromatographic based assays were reportedly used in combination with tandem mass spectrometry (LC–MS/MS). Sample preparation for the LC–MS/MS methods

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includes liquid/liquid extraction [16–18] and solid phase extraction [19]. A validated method using HPLC coupled to UV detection for doxylamine quantification in plasma was used to evaluate the pharmacokinetics of doxylamine succinate following intranasal, oral and intravenous administration in Rats [20]. However, no other methods have been reported for the simultaneous quantification of dextromethorphan, dextrorphan and doxylamine in human plasma.

2. Methods

2.1. Chemicals and materials

Dextromethorphan hydrobromide was purchased from Sigma–Aldrich (USA). Dextromethorphan-d3, dextrorphan, dextrorphan-d4, doxylamine succinate and doxylamine-d5 were synthesized by the Procter & Gamble do Brasil (Brasil). All standards, including the deutered internal standards, were donated by Procter & Gamble do Brasil (São Paulo, SP, Brazil). All reagents and solvents were HPLC grade and purchased from a local distributor. Human Plasma samples (normal, hyperlipemic and hemolyzed) came from distinct drug free subjects and were donated by the Hemocentro of Campinas, Brazil (Campinas, Brazil). Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and then stored at approximately −20 °C until needed.

2.2. Calibration standards and quality control

Stock solutions of dextromethorphan, dextrorphan and doxylamine (Fig. 1) were prepared in methanol/water (50/50, v/v) at 1.0 mg mL^{-1}. Work solutions were prepared by serial dilutions of the stock solutions in methanol–water (50:50, v/v) to obtain a series of solutions ten times more concentrated than the calibration curve or quality control samples. Calibration curves for dextromethorphan, dextrorphan and doxylamine were prepared by spiking blank plasma with work solutions to obtain the final concentrations of 0.2, 0.5, 1, 2, 5, 20, 50 and 200 ng mL^{-1} for dextromethorphan and doxylamine, and 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng mL^{-1} for dextrorphan. The analyses were carried out in duplicate for each concentration. Quality control samples were prepared in blank plasma at concentrations of 0.2, 0.6, 18.0, and 180 ng mL^{-1} [LLOQ (lower limit of quantification), QCL (quality control at low level), QCM (quality control at medium level) and QCH (quality control at high level), respectively] for dextromethorphan and doxylamine, and 0.05, 0.15, 0.9 and 9.0 ng mL^{-1} (LLOQ, QCL, QCM and QCH, respectively) for dextrorphan. The spiked plasma samples (standards and quality controls) were extracted in each analytical batch along with the unknown samples.

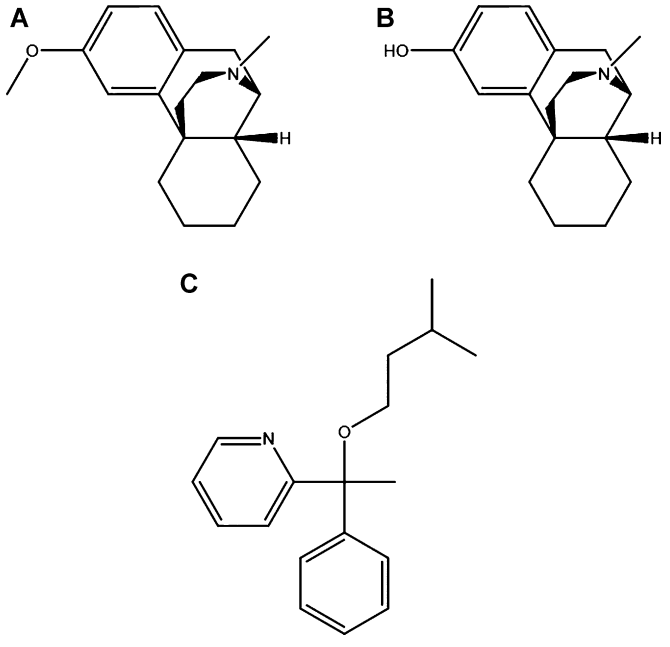

Fig. 1. Chemical structures for dextromethorphan (A), dextrorphan (B) and doxylamine (C).

2.3. Sample preparation

All frozen human plasma samples, including calibration standards, quality control and unknown samples were initially thawed at room temperature and centrifuged at 2000× *g* for 2 min at 4 °C to remove any clot or precipitate. A 500 μL of human plasma sample was pipette transferred to plastic microtubes followed by the addition of 50 μL of a mixed internal standard solution 100/100/10 ng mL^{-1} for dextromethorphan-d3, doxylamine-d5 and dexthorphan-d4, respectively in methanol (100%). After vortex mixing for 10 s, 100 μL of bicarbonate buffer 0.1 M at pH 10.5 was added and vortex-mixed for approximately 30 s. Diethylether/hexane (80/20, v/v) was added (4 mL) to all tubes, and the extraction was performed by vortex-mixing for 40 s. The upper organic phase was transferred to another set of clean glass tubes and evaporated to dryness under N_2 at 40 °C. The dry residues were dissolved with 150 μL of a solution of acetonitrile:water (50:50, v/v). Vials were capped and then placed into the autosampler.

2.4. Chromatographic conditions

After extraction 10 μL of the samples were injected into a Phenomenex Gemini® C18, 5 μm analytical column (150 × 4.6 mm i.d.) operating at room temperature. The compounds were eluted by pumping the mobile phase (acetonitrile/water/formic acid (90/9/1, v/v/v) at a flow-rate of 1.5 mL min^{-1} using a Shimadzu HPLC pump (model LC10AD, Shimadzu, Japan). The total run time was 4 min.

Under these conditions, typical standard retention times were 2.0 ± 0.8 min for dextromethorphan, dextrorphan, dextromethorphan-d3 and dextrorphan-d4, and 3.0 ± 1.0 min for doxylamine and doxylamine-d5.

2.5. Mass spectrometric conditions

MS detection was performed in the positive mode on an Applied Biosystems Sciex API 4000 tandem triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization source. Interface parameters were optimized during infusion of the compounds through the ion source operating in positive mode (ESI+) connected with the LC system and were as described in Table 1. The *m*/*z* 272.2 > 215.2 transition was monitored for dextromethorphan, the *m*/*z* 275.2 > 218.3

Table 1
Individual parameters of detection to each ion monitored at Multiple Reaction Monitoring mode.

Precursor ion > product ion (m/e)	Dwell time (s)	DP (V)	CE (eV)	CXP (V)
272.2 > 147.2	200	71	43	26
275.2 > 218.3	200	56	33	14
271.2 > 182.2	200	31	21	32
276.2 > 187.2	200	26	21	50
258.2 > 133.1	200	81	41	24
262.2 > 157.2	200	76	57	4

DP, declustering potential; CE, collision energy; CXP, collision exit potential.

transition for dextromethorphan-d3, the m/z 258.1 > 133.1 transition for dextrorphan, the m/z 262.1 > 137.1 for dextrorphan-d4, the m/z 271.2 > 182.2 for doxylamine and m/z 276.2 > 187.2 for doxylamine-d5. Data acquisition and analysis were performed using the software Analyst (version 1.4.2).

2.6. Linearity

The standard calibration curves were constructed using the peak area ratios of each analyte and IS vs. the analyte nominal concentrations of the eight plasma standards in duplicate. The concentrations used were 0.2, 0.5, 1, 2, 5, 20, 50 and 200 ng mL^{-1} for dextromethorphan and doxylamine, and 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng mL^{-1} for dextrorphan. Although chromatographic procedures are typically heteroscedastic and a linear least-square regression analysis with weighting factor of $1/x^2$ is normally more appropriate to assess the linearity, our calibration curve showed no difference in linearity using the weighting factor of $1/x$ to generate the standard calibration equation:

$$y = ax + b,$$

where y is the peak–area ratio, x the concentration, a the slope and b is the intercept of the regression line.

Since the validation procedure should confirm the ability of the method to unequivocally assess the analyte in the presence of other components that may be present (for example, impurities, degradation products and matrix components), the presence of interferences was evaluated using a blank (non-spiked sample) and a zero plasma sample (only spiked with IS).

2.7. Ion suppression

A procedure to assess the effect of ion suppression on MS/MS was performed. The experimental set-up consisted of an infusion pump connected to the system by a 'zero volume tee' before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of each analyte. The infusion pump was set to transfer (10.0 μL min^{-1}) of a mixture of analyte and internal standard in mobile phase (both 10 ng mL^{-1}). The reconstituted extract was injected into the HPLC system while the standard mixture of each individual analyte and IS in mobile phase was infused. The ion suppression was evaluated in three different matrices: normal, lipemic and hemolized plasma samples. The samples of human pooled blank plasma were extracted following the full extraction procedure. In this system, any ion suppression would be observed as a depression of the MS signal.

2.8. Recovery

The recovery was evaluated by calculating the mean of the response of five replicates of each QCL, QCM and QCH concentration for each analyte and dividing the extracted sample mean response by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. Comparison with the unextracted samples, spiked on plasma residues obtained after performing the full extraction process in blank plasma samples, was done in order to eliminate matrix effects, giving a true recovery.

2.9. Precision and accuracy

Precision and accuracy of this method were evaluated using three different batches of quality control samples of each analyte, also including the lowest limit of quantification (LLOQ). For intra-batch assay precision and accuracy, eight replicates of quality control samples were assayed all at once within a day to obtain %CV and accuracy values. The inter-batch assay precision and accuracy were determined by analyzing mean values of quality control samples from three plasma batches, performed at different days.

2.10. Sensitivity

The lower limit of quantification (LLOQ) was determined for each analyte based on two criteria: (a) the analyte response at LLOQ had to be at least five times baseline noise; (b) the analyte response at LLOQ being determined with sufficient precision and accuracy, i.e., precision of 20% and accuracy of 80–120%. Calculations were based on six replicates of three blank plasma batches.

2.11. Stability

Stability of dextromethorphan, dextrorphan and doxylamine was assessed in five replicates of plasma spiked with each analyte at QCL, QCM and QCH. To evaluate the stability of all three analytes after different conditions, all samples were extracted and further compared to fresh prepared ones at equivalent concentration.

Freeze–thaw stability was evaluated after three freeze–thaw cycles of −20 °C during 24 h. In each cycle, frozen samples were allowed to thaw at controlled ambient temperature (22 °C) and were subsequently refrozen for 24 h. Aliquots of all samples were quantified at the end of the third freeze–thaw cycle.

To evaluate the short term storage stability the low and high QCs samples were thawed at room temperature (22 °C). All samples remained on the bench top for 6 h, a time exceeding the maximum period of time expected for routine sample preparation. The long term storage stability was determined after storage for 20 days at −20 °C. Post-processing stability was evaluated after extraction of QC samples and storage in the autosampler at 8 ± 2 °C for at least 48 h.

Dextromethorphan, dextrorphan and doxylamine stock and work solutions were prepared and stored at 4 ± 6 °C. In this case, sample aliquots of five replicates of low, medium and high QCs levels were evaluated after 25 days for stock solution and 24 h for work solution stability. Similarly to plasma spiked samples, results were compared to fresh prepared solutions at corresponding concentrations.

2.12. Pharmacokinetics and statistical analysis

The usefulness of the analytical method developed here was applied to evaluate the pharmacokinetics of a suspension formulation containing the association of dextromethorphan hydrobromide and doxylamine succinate in healthy volunteers. The first-order terminal elimination rate constant (ke) was estimated by linear regression from the points describing the elimination phase in a log-linear plot. Half-life ($T_{1/2}$) was derived from this rate constant ($T_{1/2} = \ln(2)\text{ke} - 1$). The areas under the analytes plasma concentrations vs. time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear-log trapezoid rule. Extrapolation of these areas to infinity ($AUC_{0-infinite}$) was done by adding the value C_{last}/ke to the calculated AUC_{last} (where C_{last} = the last detectable concentration). The C_{max} (the maximum concentration) and T_{max} (time when the C_{max} is reached) were also calculated from the dextromethorphan, dextrorphan and doxylamine plasma concentrations vs. time curves.

Software used included WinNonLin Professional Network Edition (v. 3.2) in single compartmental model for extra vascular administration, Microsoft Excel (v. 7.0) and GraphPad Prism (v. 3.02).

Twenty four healthy volunteers of both sexes aged between 18 and 50 years and index of corporal mass within 15% of the normal index were selected for the study after assessment of their

Table 2
Mean recovery and CV (%) results for the dextromethorphan, dextrorphan, doxylamine and deutered internal standards extraction method.

Compound	QCL		QCM		QCH	
		CV (%)	Mean recovery (%)	CV (%)	Mean recovery (%)	CV (%)
Dextromethorphan	83.7	6.1	78.3	9.5	84.7	3.1
Dextrorphan	97.9	0.7	81.5	5.7	87.2	2.6
Doxylamine	76.7	6.8	72.5	10.0	66.0	5.4
Dextromethorphan-d3	96.5	4.9	98.7	4.2	101.7	4.8
Dextrorphan-d4	94.8	4.1	96.4	5.9	99.0	3.4
Doxylamine-d5	95.5	6.6	99.5	4.2	103.1	4.8

LLOQ, lower limit of quantification; QCL, quality control at low level; QCM, quality control at medium level; and QCH, quality control at high level.
LLOQ = 0.05 ng mL^{-1}; QCL = 0.15 ng mL^{-1}; QCM = 0.9 ng mL^{-1}; QCH = 9.0 ng mL^{-1}.

health status by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, uric acid, hemoglobin, hematocrit, total and differential white cell counts, routine urinalysis and pregnancy test βHCG. All subjects were negative for HIV, HCV and HBV. All subjects gave written informed consent and the study was conducted in accordance with the revised Declaration of Helsinki, the rules of Good Clinical Practice (ICH-GCP) and the Resolution Nos. 196/96 and 251/97 of National Health Council – Health Ministry, Brazil. The clinical protocol was approved by the Research Ethics Committee of Universidade Estadual de Campinas – UNICAMP, São Paulo, Brazil.

The volunteers entered the Clinical Pharmacology Unit 10 h before drug administration and left the unit 14 h after sampling.

After time 0 sampling, each volunteer received a single dose of a suspension containing dextromethorphan hydrobromide and doxylamine succinate (12.5 mg of doxylamine and 30 mg of dextromethorphan). The volunteers were then fasted for 4 h, after which period a standard lunch was served. No other food was permitted during the "in-house" period and liquid consumption was allowed ad libitum after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and cola). The subjects were monitored throughout the study and the formulations were considered to be well tolerated. Blood samples were collected by indwelling catheter into EDTA containing tubes before dosing and 30, 45 min and also 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 16, 24, 36 and 48 h post-dosing. The blood samples were centrifuged at 2000 × *g* for 10 min at 4 °C and the plasma stored at −20 °C until analyzed for the drug content.

3. Results

3.1. Linearity and specificity

The regression method used for the calibration curves was $y = a + bx$ from 0.2 to 200 ng mL^{-1} for dextromethorphan and doxylamine and 0.05 to 10 ng mL^{-1} for dextrorphan. Correlation coefficient ranged from 0.9995 to 0.9999.

The retention times for all compound, including analytes and IS were 2.0 ± 0.8 min for dextromethorphan, dextrorphan, dextromethorphan-d3 and dextrorphan-d4, and 3. 0 ± 1.0 min for doxylamine and doxylamine-d5.

The specificity evaluation showed that the presence of interfering peaks at the same retention time of the drug were less than 20% of the LLOQ response, when analyzing the blank normal plasma, and the two other batches of hemolyzed and hyperlipemic plasma. The response for the interfering peaks at the retention time of the internal standards was also less than 20%, considering the response in the concentration used. Furthermore, blank plasma samples from all volunteers were run before unknown sample quantification, showing a clear chromatogram. The main reason was the clean liquid–liquid extraction besides the high selectivity of the Multiple Reaction Monitoring (MRM) mode on LC–MS–MS spectrometer. Therefore, the high selectivity of the method was confirmed by both drug and IS, as no endogenous peaks were seen at the analytical conditions previously described. No significant interference at the retention time of analyzed compounds was found, as showed on the Zero and LLOQ chromatograms presented in Figs. 2–4. The signal-to-noise ratio was higher than 7 units for all compounds.

3.2. Recovery

Absolute recoveries for dextromethorphan, dextrorphan and doxylamine and the IS were evaluated. Considering the three levels of QC evaluated, the mean recoveries were 71.7 for doxylamine, 82.2 for dextromethorphan and 88.9 for dextrorphan. The CV (%) varied between 0.7% and 10.0% (Table 2).

3.3. Accuracy and precision

Intra-batch precision and accuracy of the assay were measured for all analytes at each QC level, including the LLOQ. Calculated intra-batch precision and accuracy (%CV) of the method for the QC samples ranged from 2.5 to 9.5%, and 88.9 to 105.1%, respectively. Method inter-batch precision (%CV) and accuracy ranged from 6.7 to 10.3%, and 92.2 to 107.1%, respectively, as presented in Table 3. For a complete visualization of the accuracy and precision results, LLOQ values are also presented in the same table.

Table 3
Summary of % intra- and inter-batch precision and accuracy results for the quantification of Quality Control (QC) samples of dextromethorphan, dextrorphan and doxylamine in human plasma.

	Intra-batch		Inter-batch	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Dextromethorphan				
LLOQ	7.7	95.1	10.7	97.2
QCL	6.3	92.1	8.1	97.0
QCM	9.4	97.2	9.3	100.8
QCH				
Dextrorphan				
LLOQ	11.3	105.8	14.8	111.2
QCL	6.6	105.1	9.3	107.1
QCM	9.5	96.8	8.8	99.4
QCH				
Doxylamine				
LLOQ	11.4	95.6	14.2	102.8
QCL	7.2	88.9	7.6	92.2
QCM	9.4	96.6	8.9	99.6
QCH				

n = 3 for each test.
LLOQ, lower limit of quantification; QCL, quality control at low level; QCM, quality control at medium level; and QCH, quality control at high level.
LLOQ = 0.05 ng mL^{-1}; QCL = 0.15 ng mL^{-1}; QCM = 0.9 ng mL^{-1}; QCH = 9.0 ng mL^{-1}.

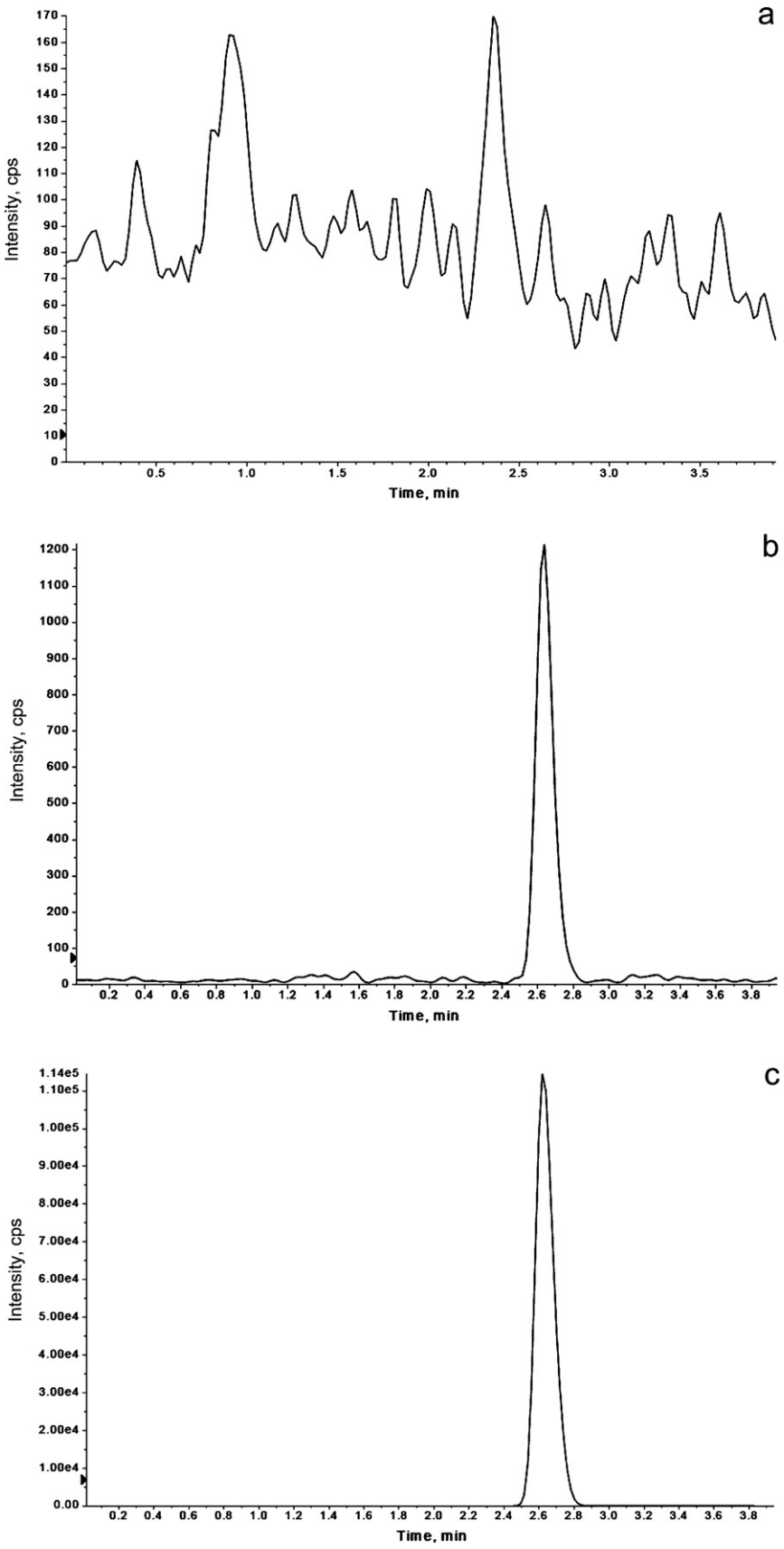

Fig. 2. (A) MRM chromatogram (272.2 > 215.2) of blank pooled human plasma for dextromethorphan. (B) MRM chromatogram of dextromethorphan spiked in human plasma at a final concentration of 0.2 ng mL^{-1}. (C) MRM chromatogram (275.2 > 218.3) of dextromethorphan-d3 spiked in human plasma at a final concentration of 10 ng mL^{-1}.

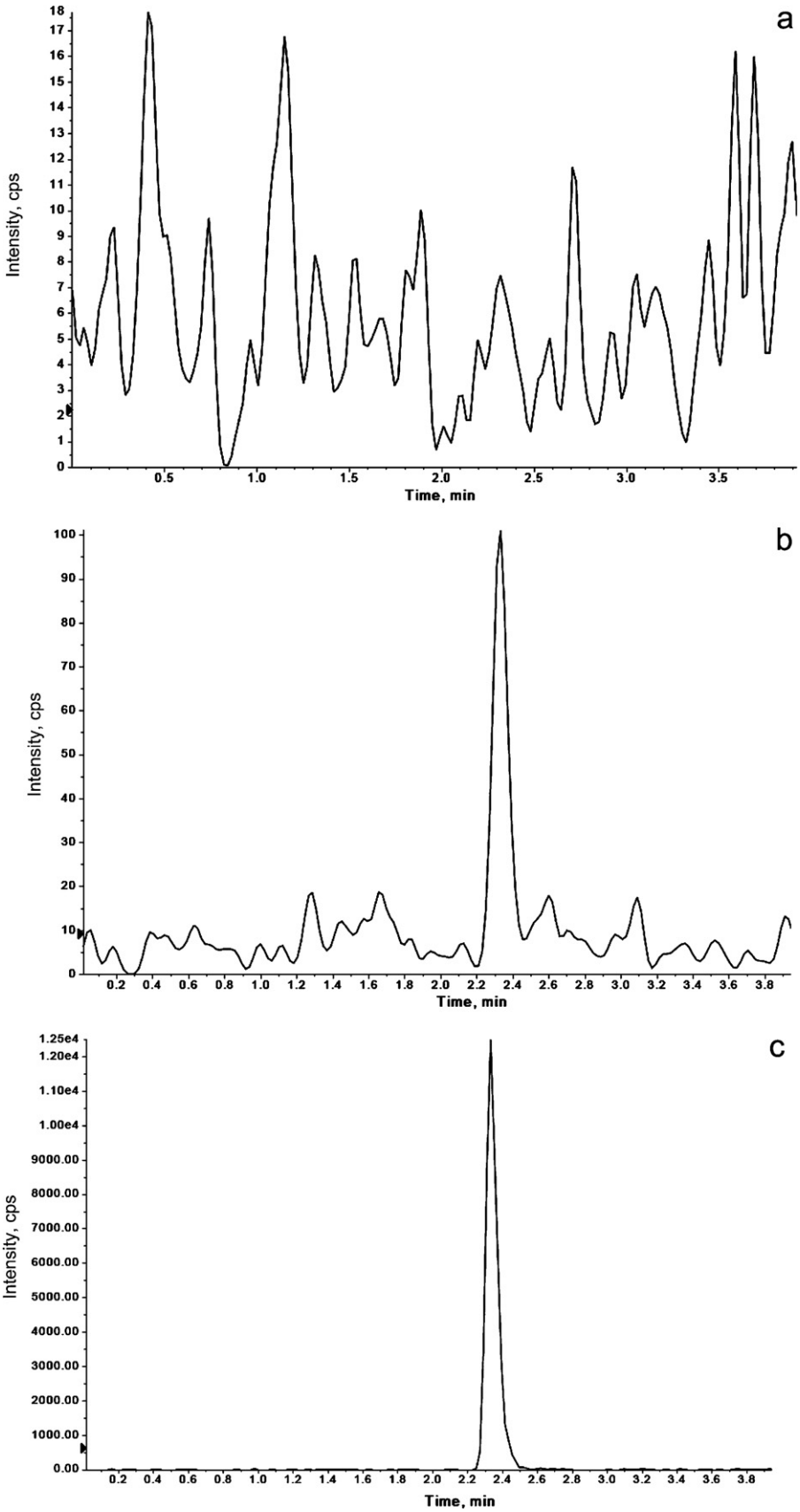

Fig. 3. (A) MRM chromatogram (258.1 > 133.1) of blank pooled human plasma for dextrorphan. (B) MRM chromatogram of dextrorphan spiked in human plasma at a final concentration of 0.05 ng mL^{-1}. (C) MRM chromatogram (262.1 > 137.1) of dextrorphan-d4 spiked in human plasma at a final concentration of 1.0 ng mL^{-1}.

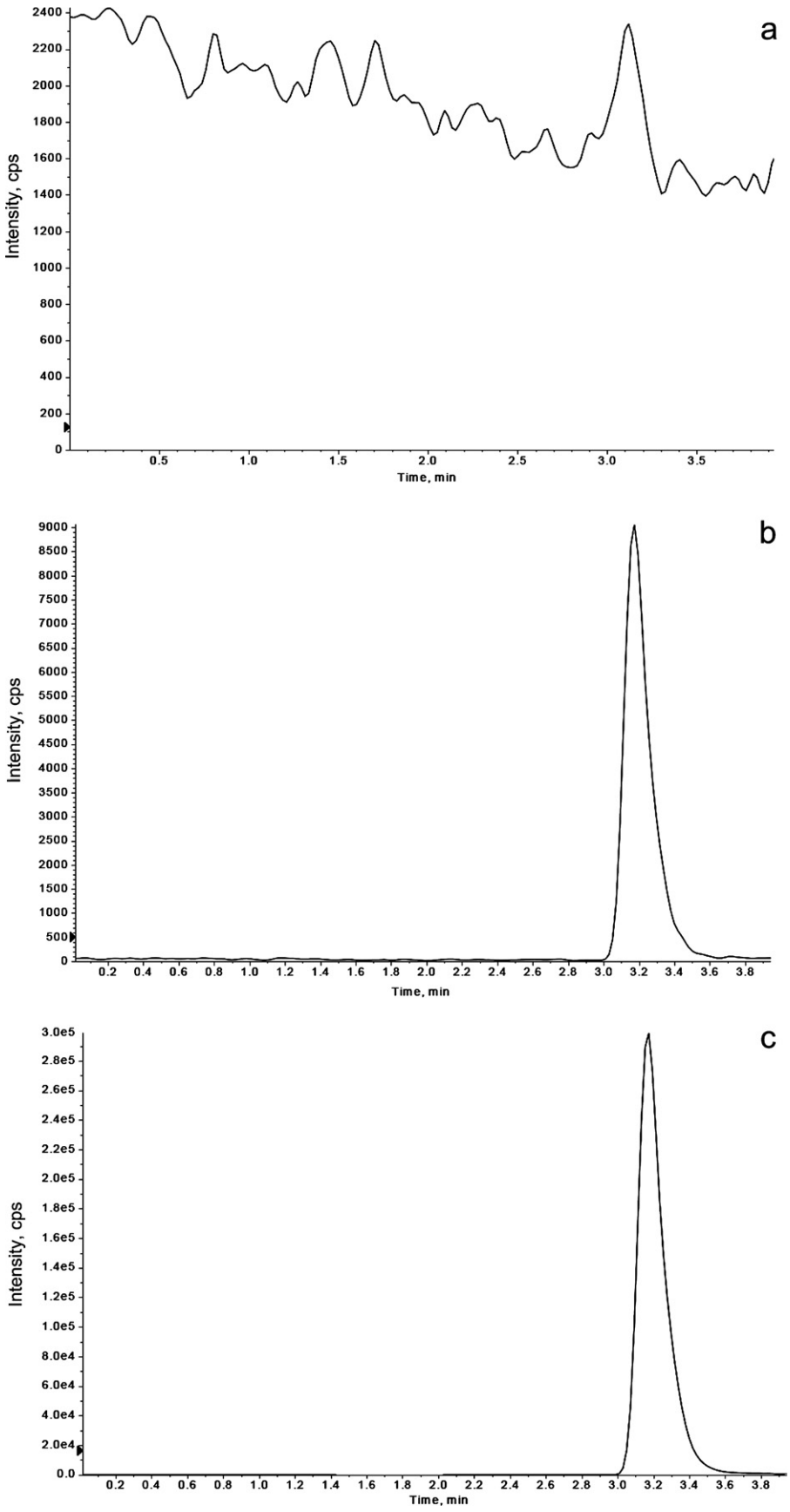

Fig. 4. (A) MRM chromatogram (271.2 > 182.2) of blank pooled human plasma for doxylamine. (B) MRM chromatogram of doxylamine spiked in human plasma at a final concentration of 0.2 ng mL^{-1}. (C) MRM chromatogram (276.2 > 187.2) of doxylamine-d5 spiked in human plasma at a final concentration of 10 ng mL^{-1}.

Table 4
Stability tests for doxylamine, dextromethorphan and dextrorphan.

	Post-processing stability test (48 h at 8 ± 2 °C)					
	QCL		QCM		QCH	
	Initial	Final	Initial	Final	Initial	Final
Doxylamine						
Mean (ng mL^{-1})	0.589	0.589	17.2	17.0	191.0	182.0
CV (%)	10.7	11.3	1.8	1.4	5.7	13.7
Variation	0		−1.3		−4.3	
Dextromethorphan						
Mean (ng mL^{-1})	0.63	0.64	17.1	17.1	178.0	175.0
CV (%)	6.6	8.5	2.7	1.2	3.1	5.7
Variation	1.6		−0.1		−1.7	
Dextrorphan						
Mean (ng mL^{-1})	0.155	0.169	0.869	0.88	9.36	9.21
CV (%)	8	7.9	2.9	4.2	4	4.7
Variation	8.8		1.3		−1.6	
Freeze-and-thaw stability test (three cycles)						
Doxylamine						
Mean (ng mL^{-1})	0.602	0.641	16.0	15.0	175.0	172.0
CV (%)	11.9	19.5	1.6	0.6	16.6	14.6
Variation	6.5		−5.9		−1.4	
Dextromethorphan						
Mean (ng mL^{-1})	0.537	0.592	15.6	15.0	157.0	162.0
CV (%)	10.2	17.2	2.3	0.9	8	8.8
Variation	10.3		−3.7		3.2	
Dextrorphan						
Mean (ng mL^{-1})	0.177	0.159	0.832	0.771	8.4	8.54
CV (%)	23.5	14.3	0.7	2.5	6.5	8.6
Variation	−10.1		−7.3		1.7	
Short-term stability test (6 h at room temperature)						
Doxylamine						
Mean (ng mL^{-1})	0.602	0.662	16.0	16.6	175.0	164.0
CV (%)	11.9	20.2	1.6	8.0	16.6	5.1
Variation	10		3.6		−6.2	
Dextromethorphan						
Mean (ng mL^{-1})	0.537	0.62	15.6	16.1	157.0	151.0
CV (%)	10.2	16.9	2.3	6.9	8	1.9
Variation	15.5		3.2		−4.1	
Dextrorphan						
Mean (ng mL^{-1})	0.176	0.156	0.832	0.848	8.4	8.07
CV (%)	23	10	0.7	5.7	6.5	3.1
Variation	−11.6		2		−4	
Long-term stability test (20 days at −20 °C)						
Doxylamine						
Mean (ng mL^{-1})	1.06	1.18	22.2	22.1	235.0	225.0
CV (%)	30.2	35.5	2.7	2.7	7.1	10.7
Variation	12		−0.6		−4.4	
Dextromethorphan						
Mean (ng mL^{-1})	0.836	0.927	21.8	21.9	254.0	243.0
CV (%)	12.2	17.2	2.9	3.3	6.8	10.1
Variation	10.9		0.3		−4.3	
Dextrorphan						
Mean (ng mL^{-1})	0.21	0.223	1.13	1.1	13.1	12.5
CV (%)	3.1	14	5.7	6.8	6.1	11.6
Variation	6.2		−2.7		−4.7	

$n = 5$ for each test. QCL = 0.6 ng mL^{-1}; QCH = 18.0 ng mL^{-1}; QCH = 180 ng mL^{-1} for dextromethorphan and doxylamine. QCL = 0.15 ng mL^{-1}; QCH = 0.9 ng mL^{-1}; QCH = 9.0 ng mL^{-1} for dextrorphan.

All the results were within the acceptance criteria for precision and accuracy, i.e., deviation values were within ±15% of the nominal values, except for LLOQ, which could show a ±20% deviation.

3.4. Stability

The stability tests performed (Table 4), indicated no significant degradation under the conditions described above, including the freeze and thaw, short-term at room temperature and the long term (20 days) tests. After the freeze and thawing procedure, the maximum variations observed were 10.3, 10.1 and 6.5% for dextromethorphan, dextrorphan and doxylamine, respectively.

Analysis of spiked plasma samples kept at room temperature for 6 h (short-term stability) did not show any significant variation between fresh and stored samples. The maximum variations observed were 15.5, −11.0 and 10.0% for dextromethorphan, dextrorphan and doxylamine, respectively. The maximum variations were always observed with the QCL samples.

Long-term stability was also assessed after 20 days at −20 °C. Under this condition only a slight, not significant decrease was observed. The maximum differences between fresh and frozen samples were 10.9, 6.2 and 12.0% for dextromethorphan, dextrorphan and doxylamine, respectively.

After 48 h at in the autosampler (post-processing stability), the maximum variations between fresh and frozen samples were −1.7, 8.8 and −4.3% for dextromethorphan, dextrorphan and doxylamine, respectively. The HPLC–MS/MS method described herein for these compounds quantification in human plasma is applicable to analytical runs lasting 48 h (or less) as observed in this post-processing tests.

Stability tests performed indicated that there was no significant degradation of the work solution at 4 ± 6 °C. Additionally, the stock solution stability was confirmed after 25 days at the same temperature conditions. Considering both solutions, the maximum variations observed were 8.0, −1.4 and 6.9% for dextromethorphan, dextrorphan and doxylamine, respectively.

3.5. Pharmacokinetics

The mean dextromethorphan, dextrorphan and doxylamine plasma concentrations vs. time curves were obtained after a single oral dose of a formulation containing 30 mg of dextromethorphan hydrobromide and 12.5 mg of doxylamine succinate (Fig. 5). Table 5 shows the values for pharmacokinetic parameters. Analytes peak plasma concentrations (C_{max}) values and the time values achieved (T_{max}) were similar to those reported in the literature by [16,18]. The high sensitivity of the method allows for a precise evaluation of pharmacokinetic parameters, mainly of ke, $T_{1/2}$ and AUC_{inf}.

4. Discussion

A simple, rapid and specific method for simultaneously quantifying dextromethorphan and dextrorphan simultaneously with doxylamine in human plasma was fully validated. The combination liquid chromatography/mass spectrometry is currently accepted as being a powerful means of determining organic molecules from complex biological matrices [21–23]. The selectivity and sensitivity of LC–MS/MS allowed analysis times to be reduced, such that sample preparation time often exceeds the analysis time of samples. Results of sample extraction procedure showed that overall recovery was very high and reproducible for all the analytes. Besides the recovery variation among the three analytes, the specific recovery for each one presented a coefficient of variation <10% and was very constant at the three levels of QC evaluated.

The LLOQ of 0.2 ng mL^{-1} for dextromethorphan and 0.05 ng mL^{-1} for dextrorphan is only of intermediate sensitivity compared to the literature, but is adequate for pharmacokinetics studies and could be further improved by sample concentration if required. While the on-line SPE method does provide an adequate solution for smaller batches of samples where higher LLOQs are acceptable, it is not a practical and economical solution when considering the demanding needs of the current assay requiring simultaneous quantification of these three structurally diverse analytes in more than 6200 study samples.

The method described by Eichhold et al. [17] also used a simple LLE extraction procedure. Even presenting a much lower LLOQ (5 pg mL^{-1}) for both dextromethorphan and dextrorphan, a significantly higher sample volume (1.0 mL) was used for the compounds extraction compared to the 0.5 mL used in our method. The sample volume is one of the critical factors in a PK study with human subjects. Two others recent methods based on LC–MS/MS technology described the simultaneous quantification of dextromethorphan and dextrorphan with lower LLOQ [16,18]. However, these methods used complicated, expensive using a not thus far popular automated or semi-automated systems for sample extraction followed by LC–MS/MS analysis. Basically, these methods used a semi-automated liquid handling systems to perform the majority of the sample manipulation during a liquid/liquid extraction (LLE) of the analytes from human plasma.

Table 5
Pharmacokinetic parameters obtained from 24 volunteers after administration of 30 mL of a suspension containing 30 mg of dextromethorphan hydrobromide and 12.5 mg of doxylamine succinate.

Pharmacokinetics parameters	Mean	SD	SE	Min	Median	Max
Dextromethrophan						
$AUC_{0-infinite}$ ([ng h] mL^{-1})	10.76	9.22	1.92	2.13	8.08	36.54
AUC_{last} ([ng h] mL^{-1})	7.78	8.34	1.67	0.00	4.28	31.69
C_{max} (ng mL^{-1})	1.42	1.29	0.26	0.00	0.85	5.19
ke (1 h^{-1})	0.14	0.06	0.01	0.06	0.14	0.31
$T_{1/2}$ (h)	5.79	2.38	0.50	2.27	5.10	11.44
T_{last} (h)	11.04	6.75	1.38	3.00	10.00	24.00
T_{max} (h)	1.83	0.72	0.15	1.00	1.75	4.00
Dextrorphan						
$AUC_{0-infinite}$ ([ng h] mL^{-1})	27.07	11.82	2.36	10.45	26.94	63.25
AUC_{last} ([ng h] mL^{-1})	26.55	11.80	2.36	9.95	26.55	62.71
C_{max} (ng mL^{-1})	6.63	2.86	0.57	1.76	6.04	12.80
ke (1 h^{-1})	0.18	0.04	0.01	0.10	0.18	0.25
$T_{1/2}$ (h)	4.09	0.96	0.19	2.80	3.78	6.90
T_{last} (h)	22.58	4.46	0.89	16.00	24.00	36.00
T_{max} (h)	1.25	0.60	0.12	0.50	1.00	3.00
Doxylamine						
$AUC_{0-infinite}$ ([ng h] mL^{-1})	669.66	168.31	34.36	404.94	685.89	1082.24
AUC_{last} ([ng h] mL^{-1})	622.94	136.61	27.89	392.63	652.91	835.52
C_{max} (ng mL^{-1})	46.57	8.76	1.79	29.30	46.60	61.50
ke (1 h^{-1})	0.07	0.01	0.002	0.05	0.06	0.09
$T_{1/2}$ (h)	10.77	1.71	0.35	7.80	10.74	13.89
T_{last} (h)	46.34	6.90	1.41	24.00	48.00	50.10
T_{max} (h)	1.71	0.98	0.20	0.50	1.50	4.00

SE, standard error; SD, standard deviation.

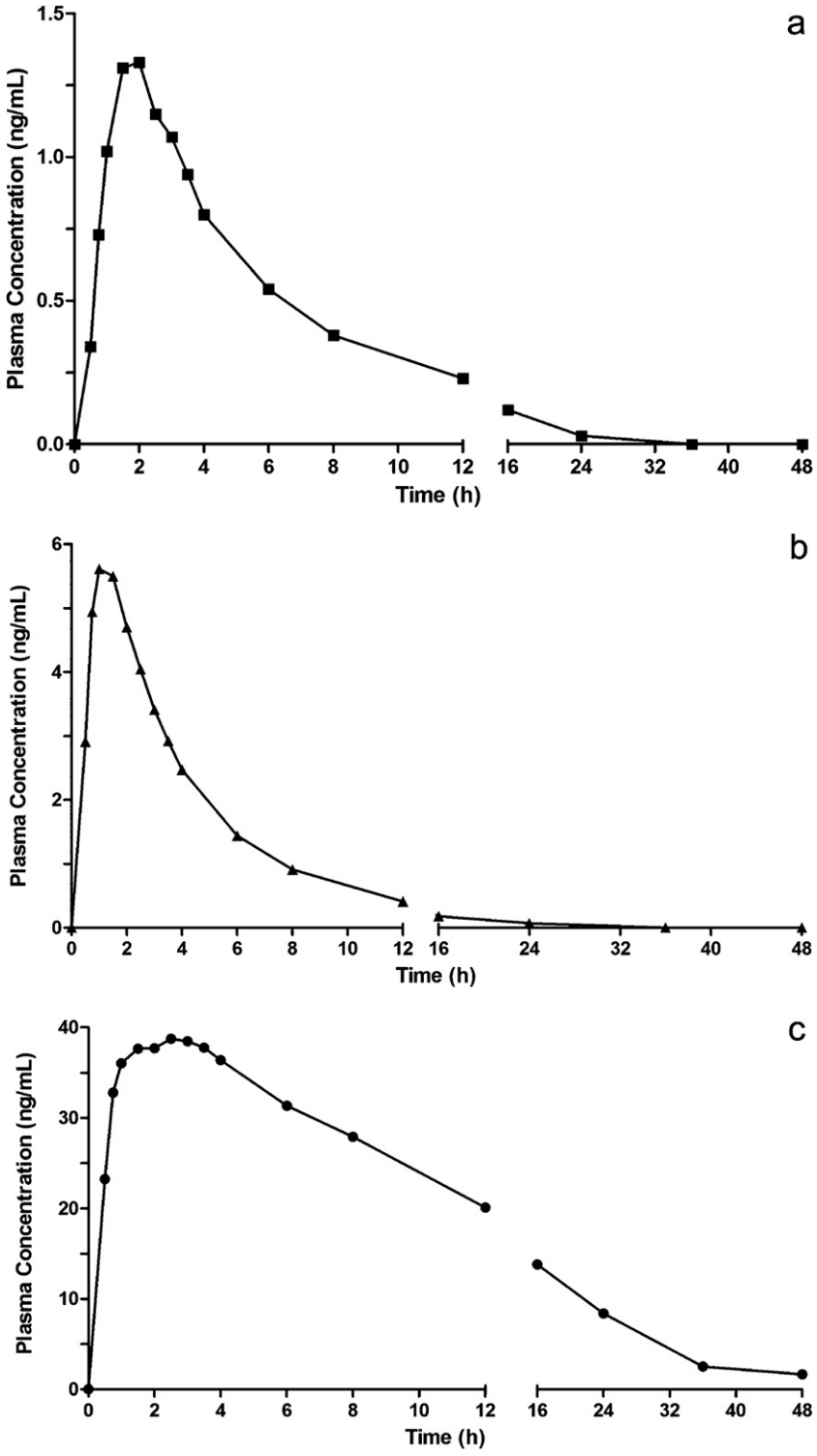

Fig. 5. Mean dextromethorphan (a), dextrorphan (b) and doxylamine (c) plasma concentrations vs. Time profile obtained after the single 30 mL oral administration of a suspension containing 30 mg of dextromethorphan hydrobromide and 12.5 mg of doxylamine succinate in 24 human healthy volunteers. The concentration values at $t = 36$ h in "a" and "b" were below the limit of quantification. For that reason, the mean value is between the 0.0 ng mL^{-1} and the LLOQ (0.05 ng mL^{-1}).

The method described by Liu et al. [24] also used a simple LLE extraction procedure but analyzed only the dextrorphan concentration with a much higher LLOQ (0.2 ng mL^{-1}). On the other hand, the LC–MS/MS method described by Eichhold et al. [19] analyzed only the dextromethorphan concentration (LLOQ 50 pg mL^{-1}) after a solid-phase extraction.

There are few pharmacokinetic studies of doxylamine succinate in humans subjects describing the details of a doxylamine quantification method [25,26]. The work published by Nulman and Koren [26] briefly describes a validated LC–MS/MS method for doxylamine, pyridoxine, and pyridoxal quantification in plasma. The LLOQ is 5 times higher than the 0.2 described in this work. As

expected, our method for doxylamine quantification is very superior to the HPLC coupled to a UV detector [20].

This is the first method for simultaneous quantification of dextromethorphan, dextrorphan and doxylamine in human plasma. Based on the assay procedure results, mainly specificity, accuracy and precision, this method agrees with the requirements for high sensitivity, specificity and high sample throughput to be applied in PK clinical studies such as bioequivalence or for the routine evaluation. This assay has been tested in a clinical setting and allows for the accurate measurement of plasma dextromethorphan and its metabolite dextrorphan and doxylamine concentration time curves. The plasma concentrations and the main pharmacokinetics parameters C_{max} and AUC values observed in this study for both dextromethorphan and dextrorphan are consistent with the published literature [16,18].

5. Conclusion

The combination LLE with isocratic elution LC–MS/MS pumping the mobile phase acetonitrile/water/formic acid (90/9/1, v/v/v) provides a very reliable, simple and rugged methodology for the high-throughput analysis of plasma samples to quantify dextromethorphan, dextrorphan and doxylamine. Our method produced very clean extracts and provided excellent ruggedness of the LC–MS/MS analysis with virtually no ionization suppression and high recovery of all analytes. The use of stable-labeled internal standards for each analyte facilitates accurate and precise quantification with a single set of extraction and LC conditions. This method proved sensitive enough to allow for the quantification of the analytes for a 48 h period following oral dosing with a commercially available cough formulation. Assay accuracy and precision were monitored through recovery of QC samples and were shown to be excellent with average accuracies within the acceptance criteria. The PK results showed the usefulness of this method allowing the daily analysis of hundreds of samples, while only small quantities of plasma and solvent are consumed.

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