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Simultaneous determination of dextrorphan and guaifenesin in human plasma by liquid chromatography with fluorescence detection

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Abstract

A sensitive liquid chromatographic (LC) method was developed and validated for the simultaneous determination of dextrorphan and guaifenesin in human plasma using fluorescence detection. Dextrorphan and guaifenesin were extracted from plasma by a liquid–liquid extraction procedure using chloroform containing laudanosine as the internal standard. A cyano column (15 cm × 46 mm i.d., Spherisorb 5-CN) and a mobile phase containing acetonitrile–triethylamine–distilled water (10:1:89, v/v/v) (pH 6) were used. The concentration–response relationship for dextrorphan was found to be linear over a concentration range of 23–515 ng ml^{-1} with a lower limit of detection of 20 ng ml^{-1}; the accuracy of the method would fall (95% confidence limit) within 9.53% and 11.07% of the true value for the inter-and intra-day, respectively; the inter- and intra-day precision, as measured by RSD, ranged from 1.88% to 30.07% (mean 2.28%) and from 4.69% to 7.51% (mean 5.67%) over the dynamic concentration range of the method (33–326 ng ml^{-1}). The concentration–response relationship for guaifenesin was found to be linear over a concentration range of 181–8136 ng ml^{-1} with a lower detection limit of 30 ng ml^{-1}; the accuracy of the method would fall (95% confidence limit) within 9.78% and 8.04% of the true value for the inter- and intra-day, respectively; the inter- and intra-day precision, as measured by the RSD, ranged from 2.55 to 6.07% (mean 3.90%) and from 3.12 to 3.90% (mean 3.52%) over the dynamic concentration range of the method (435–6430 ng ml^{-1}). The relative percentage recovery of dextrorphan, guaifenesin, and laudanosine was found to be 96%, 94%, and 88%, respectively. Benchtop and storage stability of the plasma samples were found to be adequate. In addition, the frozen plasma samples were submitted to three filter freeze/thaw cycles without significant change in the stability of guaifenesin and dextrorphan.

Keywords: Dextromethorphan; Dextrorphan; Guaifenesin; Human plasma; LC

1. Introduction

Dextromethorphan is a widely used antitussive agent. Following oral administration, it is rapidly and extensively metabolized. The major metabolite is dextrorphan and its glucuronic and sulfate metabolites [1–3]. This rapid metabolism results in extremely low dextromethorphan plasma concentrations which are very difficult to quantify. Thus, the bioavailability of dextromethorphan can be established by measuring the formation of dextrorphan, the major metabolite of dextromethorphan. Numerous analytical methods based on paper chromatography [4], thin layer chromatography [5,6], calorimetry [7], spectrophotometry [8,9], gas chromatography [10–16] and liquid chromatography [3,17–36] were developed to analyze dextrorphan and/or

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guaifenesin. However, no specific method has been reported to determine dextrorphan and guaifenesin simultaneously in physiological fluids. The objective of this study was to develop and validate liquid chromatographic (LC) method capable of measuring both dextrorphan and guaifenesin in human plasma, with the purpose of establishing the pharmacokinetic profile of dextrorphan and guaifenesin after oral administration of sustained release formulations to normal male human volunteers and bioavailability studies.

2. Experimental

2.1. Reagents and chemicals

Dextrorphan, dextromethorphan, 3-hydroxymorphinan, and 3-methoxymorphinan were obtained from Hoffman-La-Roche Inc. (Nutley, NJ, USA). Guaifenesin was obtained from Lee Laboratories. Laudanosine used as internal standard was obtained from Aldrich (Milwaukee, WI, USA). β-Glucuronidase (Helix Pomatia, type H-1) was obtained from Sigma (St. Louis, MO, USA). Acetonitrile (EM Science, Gibbstown, NJ, USA) and orthophosphoric acid (Fisher Scientific Co., Fair Lawn, NJ, USA) were HPLC grade. Triethylamine (EM Science), chloroform (Mallinckrodt), and sodium carbonate (MCB Reagents) were of analytical grade. Heparinized human plasma was obtained from Interstate Blood Bank (Memphis, TN, USA). All reagents were used as received.

2.2. Chromatographic conditions

The chromatograph consisted of a solvent delivery pump (Beckman, model 100A), Spherisorb 5-CN analytical column (15 cm × 4.6 mm i.d.) (Phenomenex Inc., Torrance, CA, USA), a fluorescence detector (Shimadzu, model RF 535), and autoinjector (Shimadzu, model Sil-9A), and an integrator (Shimadzu, Chromatopac CR 501). The mobile phase composition consisted of acetonitrile–triethylamine–and distilled-deionized water (10:1:89, v/v/v), adjusted to pH 6 with orthophosphoric acid. The mobile phase was then filtered through a 0.44 μm pore nylon membrane filter, and deaerated by sonication under reduced pressure. The flow rate was maintained at 1 ml min^{-1}. The wavelength of the fluorescence detector was set at 280 and 315 nm for excitation and emission wavelength, respectively. The detector sensitivity and response were set to low and medium, respectively.

2.3. Standard solutions

Dextrorphan standard solutions

Standard solutions of dextrorphan were prepared in acetonitrile containing approximately 0.6, 1.5, and 6.0 μg ml^{-1} of dextrorphan.

Guaifenesin standard solutions

Standard solutions of guaifenesin were prepared in distilled-deionized water containing approximately 12.0, 30.0 and 120.0 μg ml^{-1} of guaifenesin.

Internal standard solution

A standard solution of internal standard, laudanosine was prepared in acetonitrile to yield a concentration of 2.58 μg ml^{-1}.

2.4. Sample preparation

Appropriate volumes of stock solutions of dextrorphan and guaifenesin were pipetted into a 15 ml glass culture tube followed by the addition of 1 ml of human plasma, to yield standards of dextrorphan and guaifenesin in the concentration range of 23–515 ng ml^{-1} and 181–8136 ng ml^{-1}, respectively.

The plasma standard was mixed with 100 μl of internal standard solution and 500 μl of saturated sodium carbonate solution, and mixed with a vortex mixer for 10 s. To this solution was added 5 ml of chloroform and mixing was continued for an additional 10 s with a vortex mixer. The extraction with chloroform proceeded for 40 min using a rocking mixer.

The solution was then centrifuged for 25 min at 2000*g*. The aqueous layer was discarded by aspiration and the chloroform layer was transferred to a clean glass culture tube. The chloroform was evaporated to dryness under a stream of nitrogen. The residur was reconstituted in 400 μl of mobile phase, of which 300 μl was injected into the column by means of an automated injector.

2.5. Enzymatic hydrolysis of guaifenesin

Plasma samples obtained after oral administration of 10 ml Glytuss® every 4 h to normal

healthy male volunteers were used to find the most efficient incubation time for the enzymatic hydrolysis of the guaifenesin and dextrorphan conjugates. A volume of plasma (1 ml) was incubated at 37 °C with 1 ml of 3000 units ml^{-1} of β-glucuronidase type H-1 solution, which was prepared by dissolving β-glucuronidase type H-1 in 0.1 M sodium citrate, pH 5.0 [17] for 0, 0.5, 1.5, 2, 2.5, 5, 6, 12, and 25 h. After this incubation period the samples were submitted to the procedure discussed in Section 2.4.

3. Results and discussion

3.1. Chromatography

Sample chromatograms of blank plasma, plasma spiked with dextrorphan, plasma spiked with guaifenesin, dextrorphan and internal standard (low, medium, and high concentration of dextrorphan and guaifenesin) are presented in Fig. 1. The relative retention times for dextrorphan and guaifenesin were 0.77 (dextrorphan/laudanosine) and 0.149 (guaifenesin/laudanosine), respectively. There were no interfering peaks in the chromatographic region of the analytes of interest.

3.2. Enzyme hydrolysis of guaifenesin

An incubation time of 2.0 h at 37 °C using 1 ml of the enzyme solution per ml of plasma yielded complete hydrolysis of the guaifenesin and dextrorphan conjugates.

3.3. Linearity of response

The concentration–response relationship for extracted plasma standards spiked with dextrorphan and guaifenesin was found to be linear in the concentration range of 20–515 ng ml^{-1} of dextrorphan and 181–8316 ng ml^{-1} of guaifenesin. This linear relationship was demonstrated by the coefficient of variation obtained from the daily standard curves (Table 1 and 2) used for the analysis of the "unknown"

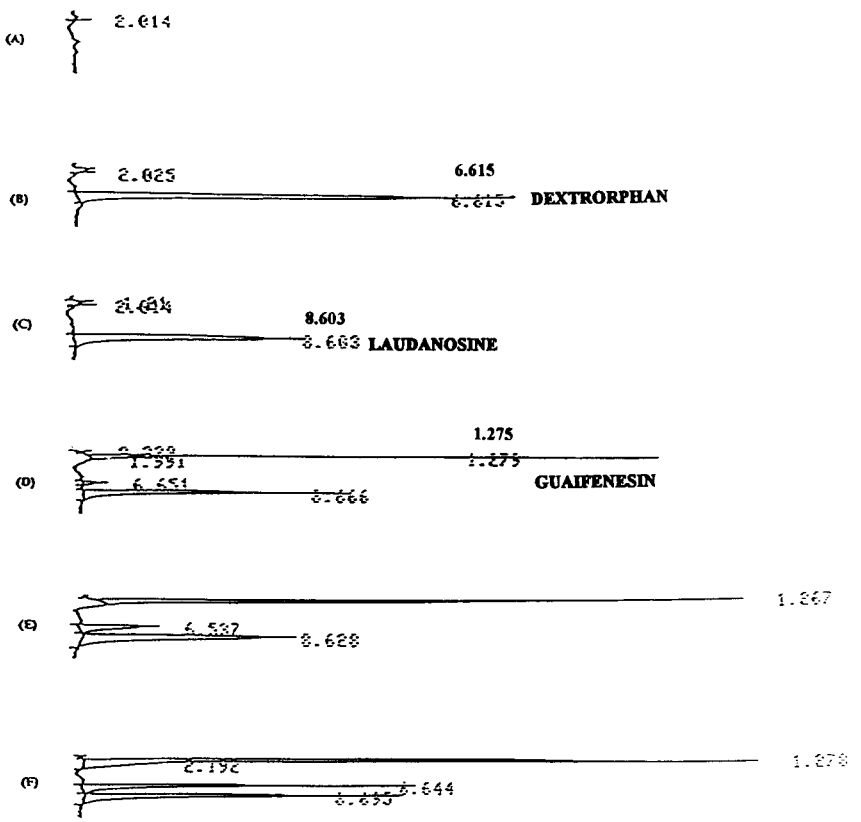

Fig. 1. Chromatograms of extracted plasma standards: (A) blank plasma; (B) blank plasma with dextrorphan; (C) blank plasma with internal standard (laudanosine); (D) plasma standard (dextrorphan 33.05 ng ml^{-1}, guaifenesin 435.6 ng ml^{-1}); (E) plasma standard (dextrorphan 93.6 ng ml^{-1}, guaifenesin 2502.0 ng ml^{-1}); (F) plasma standard (dextrorphan 351.4 ng ml^{-1}, guaifenesin 6762.2 ng ml^{-1}).

Table 1
Linearity of response. Dextrorphan standard curves in plasma

Day	Slope	Intercept	r^2
1	0.004031	−0.02283	0.999
2	0.003475	−0.00750	0.999
3	0.003615	−0.01097	0.999
4	0.004223	−0.01836	0.999
Mean	0.003836	−0.01492	0.999
SD	0.000350	0.006954	0.000

Table 2
Linearity of response. Guaifenesin standard curves in plasma

Day	Slope	Intercept	r^2
1	0.005998	−0.12320	0.995
2	0.006859	−0.09217	0.999
3	0.006287	−0.28336	0.999
4	0.008313	−0.14765	0.999
Mean	0.006864	−0.1616	0.998
SD	0.001030	0.08429	0.002

samples (Tables 3–6). The limits of detection for dextrorphan and guaifenesin were set at 20 ng ml^{-1} and 30 ng ml^{-1} of plasma, respectively. These limits were set based on three times the signal to noise levels. In the analysis of the clinical samples, some of the plasma samples showed higher levels of guaifenesin, more than 20% of the higher value (8136 ng ml^{-1}). In these cases the reconstituted residue was diluted with more mobile phase.

3.4. Accuracy

The results obtained fom the extrapolated standard concentrations are shown in Tables 3 and 4 for dextrorphan, and in Tables 5 and 6 for guaifenesin. The precentage absolute deviations of the concentrations calculated from the true value are presented in Tables 7 and 8 for dextrorphan, and in Tables 9 and 10 for guaifenesin. On any analysis day, the percentage absolute difference from the true value, for the analysis of any extrapolated standard value from 33 to 326 ng ml^{-1} for dextrorphan and from 436 to 6429 ng ml^{-1} for guaifenesin, ranged from 2.25% to 8.63% (mean 4.28%) and 2.27% to 9.67% (mean 5.79%), respectively.

Using the deviation of absolute differences between the concentrations found and the true concentration, as well as the t-value from the two-tailed student's t-distribution table, 95% confidence limits were estimated for each concentration (Tables 11 and 12). These calculations indicate that the results of any inter-day single determination for dextrorphan in plasma would range between 4.01% and 9.53% of the true value for all concentrations over the dynamic range studied. In the case of guaifenesin, the range was found to be 6.81% to 9.78%. These calculations also indicate that the results of any intra-day single determination for dextrorphan and guaifenesin in plasma would range from 5.02% to 11.07% and from 4.46% to 8.04%, respectively.

Table 4
Dextrorphan extrapolated concentrations: intra-day

	Theoretical concentration (ng ml^{-1})		
	33.16	91.72	325.71
	37.77	96.03	306.68
	36.24	86.57	310.44
	35.09	86.60	313.36
	37.78	92.79	337.95
	37.89	90.30	339.53
	33.38	92.76	307.45
	31.82	83.11	311.28
	30.64	83.15	314.27
	33.39	89.46	339.33
	33.50	86.92	340.94
Mean	34.75	88.77	322.12
SD	2.61	4.27	15.09
RSD (%)	7.51	4.81	4.69

Table 3
Dextrorphan extrapolated concentrations measured on three separate days

Nominal concentration (ng ml^{-1})	Mean of extrapolated concentration ± SD (ng ml^{-1})			Mean ($n = 3$)	SD	RSD (%)
	Day 1	Day 2	Day 3			
33.16	34.80 ± 2.70	33.91 ± 0.47	35.18 ± 3.33	34.63	0.65	1.88
91.72	91.50 ± 3.85	90.58 ± 2.47	93.94 ± 2.85	92.01	1.74	1.89
325.71	312.34 ± 1.78	326.33 ± 9.64	307.73 ± 6.54	315.47	9.69	3.07

Table 5
Guaifenesin extrapolated concentrations measured on three separate days

Nominal concentration (ng ml^{-1})	Mean of extrapolated concentration ± SD (ng ml^{-1})			Mean ($n = 3$)	SD	RSD (%)
	Day 1	Day 2	Day 3			
435.64	452.5 ± 16.69	450.9 ± 16.69	471.9 ± 21.52	458.47	11.71	2.55
2322.9	2316.2 ± 65.4	2098.2 ± 107.5	2351.0 ± 155.7	2255.1	137.0	6.07
6428.6	6406.1 ± 237.6	6466.6 ± 285.7	6101.9 ± 498.0	6324.9	195.5	3.09

3.5. Precision and reproducibility

The precision (within-day variation of replicate determinations) and the reproducibility (day-to-day variation of these determinations) are best demonstrated using the data obtained from the analysis of extrapolated standard values (Tables 3–6). For each concentration a mean value and percentage coefficient of variation were calculated on each analysis day ($n = 4$) and over the course of a three-day study. The precision of the method (within-day variation) varied from 4.69% to 7.51% (mean 5.67%) for dextrorphan, and from 3.12% to 3.90% (mean 3.52%) for guaifenesin.

The reproducibility of the method (day-to-day variation) was determined using the variability about the mean of each concentration analyzed over the course of the validation study. Over the three analysis days, the percentage coefficient of variation in the case of dextrorphan varied from 1.88% to 3.07% (mean 2.28%, see Table 3) and for guaifenesin it varied from 2.55% to 6.07% (mean 3.90%, see Table 5).

Table 6
Guaifenesin extrapolated concentrations: intra-day

	Theoretical concentration (ng ml^{-1})		
	435.6	2323	6429
	449.0	2453	6684
	439.9	2390	6555
	445.5	2444	6657
	454.0	2471	6313
	436.5	2347	6573
	477.0	2342	6279
	468.4	2283	6159
	473.8	2334	6253
	481.6	2359	5933
	465.3	2244	6176
Mean	459.1	2634	6358
SD	16.2	74.0	247.8
RSD (%)	3.53	3.12	3.90

Table 7
Dextrorphan percentage deviation (bias) at different concentrations measured on three separate days

Nominal concentration (ng ml^{-1})	Mean deviation (%) [a]			Mean ($n = 3$)	SD
	Day 1	Day 2	Day 3		
33.16	6.97	2.25	8.63	5.95	3.31
91.72	3.15	2.28	3.13	2.85	0.50
325.71	4.11	2.44	5.52	4.02	1.54

[a] $n = 4$.

Table 8
Dextrorphan percentage deviation (bias) at different concentrations: intra-day

	Nominal concentration (ng ml^{-1})		
	33.16	91.72	325.71
	13.90	4.70	5.84
	9.29	5.61	4.69
	5.82	5.58	3.79
	12.93	1.17	3.76
	14.26	1.55	4.24
	0.66	1.13	5.61
	4.04	9.39	4.43
	7.60	9.34	3.51
	0.69	2.46	4.18
	1.03	5.23	4.68
Mean	7.12	4.62	4.47
SD	5.57	3.08	0.77

Table 9
Guaifenesin percentage deviation at different concentrations: intra-day

Nominal concentration (ng ml^{-1})	Mean deviation (%) [a]			Mean ($n = 3$)	SD
	Day 1	Day 2	Day 3		
435.64	3.88	8.24	8.34	6.82	2.55
2322.9	2.27	9.67	5.48	5.81	3.71
6428.6	9.67	3.80	7.33	4.75	2.26

[a] $n = 4$.

Table 10
Guaifenesin percentage deviation at different concentrations: intra-day

	Nominal concentration (ng ml^{-1})		
	435.6	2323	6429
	3.07	5.60	3.98
	0.97	2.90	1.97
	2.27	5.23	3.55
	4.21	6.39	1.79
	0.19	1.06	2.25
	9.49	0.81	2.33
	7.54	1.70	4.19
	8.75	0.46	2.73
	10.55	1.55	7.70
	6.81	3.42	3.93
Mean	5.38	2.91	3.44
SD	3.72	2.16	1.74

3.6. Freeze-thaw cycles

Dextrorphan and guaifenesin were found to be stable in human plasma (< 5% loss) after three freeze/thaw cycles.

3.7. Benchtop stability

Benchtop stability was performed by comparing the back calculated concentration of the quality control samples kept at ambient temperature (≈ 25 °C) for 4 h to the nominal concentrations. The stability of dextrorphan and guaifenesin ranged from 95.6% to 106.6% and from 90.5% to 105.9%, respectively.

3.8. Long term stability

Quality control samples were kept at 4 °C and room temperature for a period of 48 h. The stability of dextrorphan and guaifenesin, after 48 h at room temperature, ranged from 94.6% to 9.77% and from 93.1% to 108.8%, respectively. At 4 °C for 48 h the stability of dextrorphan and guaifenesin ranged from 94.9% to 100.3% and from 94.5% to 107.6%, respectively. The stability of dextrorphan and guaifenesin after two weeks at −20 °C was found to range from 91.1% to 107.1% and from 100.2% to 107.7%, respectively.

Table 11
Confidence level analysis of Dextrorphan and guaifenesin from data measured three times on the same day

True value	Number of observations	Mean absolute difference from true value ± S.E.	95% Confidence interval	
			(ng ml^{-1})	Deviation (%)
Dextrorphan				
33.16	12	1.97 ± 0.54	±3.16	9.53
91.72	12	2.62 ± 0.48	±3.68	4.01
325.71	12	12.10 ± 1.67	±16.78	5.15
Guaifenesin				
435.64	12	29.70 ± 5.86	±42.62	9.78
2322.93	12	134.88 ± 28.49	±197.70	8.51
6428.57	12	305.02 ± 60.08	±437.50	6.81

Table 12
Confidence level analysis of Dextrorphan and guaifenesin from data measured three times on the same day

True value	Number of observations	Mean absolute difference from true value ± S.E.	95% Confidence interval	
			(ng ml^{-1})	Deviation (%)
Dextrorphan				
33.16	10	2.36 ± 0.58	±3.67	11.07
91.72	10	4.24 ± 0.89	±6.25	6.82
325.71	10	14.57 ± 0.79	±16.36	5.02
Guaifenesin				
435.64	10	23.46 ± 5.12	±35.03	8.04
2322.93	10	67.62 ± 15.89	±103.53	4.46
6428.57	10	221.21 ± 35.38	±301.17	4.68

3.9. Recovery

The relative percentage recovery of dextrorphan and guaifenesin from plasma when compared to water ranged from 92% to 100% and from 93% to 96%, respectively. The relative percentage recovery of the internal standard (laudanosine) was found to be 88%.

4. Conclusion

The data presented in this investigation indicate that the assay procedure is reliable, sensitive, and selective for assessment of bioavailability and for clinical use.

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