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Determination of guaifenesin in human serum by capillary gas chromatography and electron capture detection

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Abstract

A method for the quantitation of guaifenesin in human serum has been developed and validated. The procedure involves liquid–liquid extraction of the serum sample in the presence of mephenesin as an internal standard, followed by derivatization and analysis using capillary gas chromatography (GC) and electron capture detection (ECD). Different solvents were tested for extraction of guaifenesin from serum. *n*-Hexane/dichloromethane (1:1, v/v) gave the highest recovery and the lowest background and was chosen as the extraction solvent. After extraction, the residue of guaifenesin was derivatized at 60 °C for 30 min, with trifluoroacetic acid anhydride (TFAA) in toluene in the presence of pyridine. Excess trifluoroacetic acid anhydride was removed using dilute solution of ammonium hydroxide. The method proved to be linear over the range of 25.0–1000 ng/ml. Recovery of guaifenesin from spiked samples was consistent, averaging 75.5% at 50.0 ng/ml with a range of 72.0–80.0% ($N = 8$ determinations) and averaging 78% at 800 ng/ml with a range of 76.0–81.0% ($N = 8$ determinations). The internal standard recovery was also consistent averaging 72.8% with a range of 67.0–76.0% ($N = 16$ determinations).

Keywords: Guaifenesin; Mephenesin; Human serum; GC; ECD

1. Introduction

Several analytical methods based on paper chromatography [1], thin layer chromatography [2], colorimetry [3], spectrophotometry [4–6], gas chromatography (GC) [7–11], and liquid chromatography [12–23] have been described in the literature for the assay of guaifenesin. However, most of these methods are for the quantitation of guaifenesin in dosages forms while the ones reported for the estimation of guaifenesin in biological fluids suffer from low sensitivity. An LC method [24] was reported for the determination of guaifenesin in human plasma in the presence of pseudoephedrine with a quantitation range of 50.0–1000 ng/ml.

The objective of this work was to develop a simple, sensitive, and rapid method for the determination of guaifenesin in human serum. Due to the higher resolving power of capillary GC as compared to HPLC and

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the sensitivity of electron capture detection (ECD), GC–ECD offered an attractive alternative for the analysis of guaifenesin.

2. Experimental

2.1. Materials and reagents

USP reference standard guaifenesin was obtained from the United States Pharmacopeial Convention, Inc. (USA). Mephenesin reagent grade was obtained from Sigma Chemical Co. (USA). High purity anhydrous trifluoroacetic acid anhydride (TFAA) and heptafluorobutyric anhydride (HFBA) were purchased from Pierce (USA).

Dichloromethane, *n*-hexane of HPLC grade were purchased from Fisher Scientific (USA) while toluene of ECD nanograde was purchased from Mallinckrodt (USA). Dry absolute pyridine was obtained from Fluka (Buchs, Switzerland). Reagent grade concentrated hydrochloric acid and concentrated ammonium hydroxide were purchased from Fisher Scientific (USA). Water used was deionized, Milli-Q Type I.

2.2. Standard solutions

Guaifenesin stock solutions of 0.5 mg/ml were made by dissolving 12.5 mg of guaifenesin in deionized water in a 25 ml volumetric flask. Diluted guaifenesin working solutions were prepared from the stock solution by diluting 1–10, 1, and 2–50 ml with deionized water, giving solutions of 50, 10.0, and 20.0 μg/ml, respectively.

A second set of diluted guaifenesin control stock solutions was prepared as described above using a second aliquot of the drug. All solutions were stored in polypropylene tubes in the dark at $-20 \pm 5\,^{\circ}C$ for up to 1 month.

Stock solution of the internal standard, mephenesin was prepared in deionized water to contain 2.0 μg/ml of mephenesin. This solution was stored in a plastic container at 4 °C for up to 2 weeks.

2.3. Samples

Guaifenesin serum standards of 0.00, 25.0, 50.0, 100, 300, 500, 800, and 1000 ng/ml were prepared by appropriate dilution of the diluted guaifenesin standard stock solutions with serum that was determined to be interference-free at the retention times of guaifenesin and the internal standard.

Guaifenesin serum controls of 75.0, 500, 800, and 1600 ng/ml were prepared by appropriate dilution of the diluted guaifenesin control stock solutions with interference-free serum.

All samples were stored in polypropylene snap-cap tubes at $-20 \pm 5\,^{\circ}C$ in aliquots appropriate for a single analytical run.

2.4. Extraction

One milliliter of sample, standard or control was transferred to a glass screw-cap culture tube using a serological pipette. To this was added 100 μl of the internal standard solution and the mixture vortexed briefly. Deionized water (100 μl) was added to the matrix and water blank samples instead of the internal standard. To the mixture, 50.0 μl of 3.0 N hydrochloric acid was added and vortexed briefly followed by 5.0 ml of *n*-hexane. The tubes were capped, shaken for 10 min at low speed and centrifuged at high speed for 5 min. The upper organic layer was discarded. To the lower aqueous layer, 5.0 ml of *n*-hexane-dichloromethane (1:1, v/v) was added. The tubes were capped, shaken at low speed and centrifuged at high speed. The upper organic layer was transferred to a clean glass tube and evaporated to dryness at 50 °C under a stream of nitrogen. The aqueous layer was extracted with another 5.0 ml of *n*-hexane-dichloromethane (1:1, v/v). Which was combined with the first extract and evaporated to dryness at 50 °C under a stream of nitrogen. The residue was redissolved in 1.0 ml of toluene and derivatized as described below.

2.5. Derivatization

To the residue dissolved in toluene was added 50 μl of 5.0 M pyridine/toluene and 300 μl of TFAA. The solution was vortexed briefly and heated in a water bath at 60 °C for 30 min. Any excess reagent was removed by adding 3.0 ml of 5% ammonium hydroxide solution, vortexed for 1 min and finally centrifuged at high speed for 3 min. The bottom aqueous layer was removed and discarded using a glass pasteur pipette.

One milliliter of toluene was added followed by 3.0 ml of Milli-Q water. The tubes were capped, vortexed, and centrifuged for 3 min. The upper toluene layer (200 μl) was transferred to a labeled autosampler vial equipped with an insert. The vials were capped and transferred to the autosampler tray for subsequent analysis.

2.6. Gas chromatography

An HP 6890 gas chromatograph (Hewlett-Packard, USA) equipped with an electron capture detector and an HP 7673 autosampler was used. The megapore capillary column was a Restek Rt-x5 (30 m × 0.53 mm, 1.0 μm). An HP 3396 A integrator was used for data collection. One microliter of the toluene extract was injected (splitless); the temperature was raised from 100 to 142 °C at 3 °C/min, then from 142 to 280 °C at 70 °C/min and held for 3 min. The injection port was kept at 250 °C while the detector temperature was 280 °C. The carrier gas was helium, ultra-high purity at 17 ml/min while the makeup gas was nitrogen at a flow rate of 48 ml/min. A chromatogram of a sample is shown in Fig. 1, together with the identification of the peaks.

2.7. Quantitative determinations

Quantiation was performed using the internal standard method. Peak height ratios of guaifenesin to the internal standard are determined for all samples, standards and controls. Sample concentrations are back-calculated from an ln/ln regression produced by standards analyzed along with the unknowns. Standards and controls were injected at different times during the analytical run in order to monitor changes in chromatographic conditions. Controls were run daily to determine day-to-day variation.

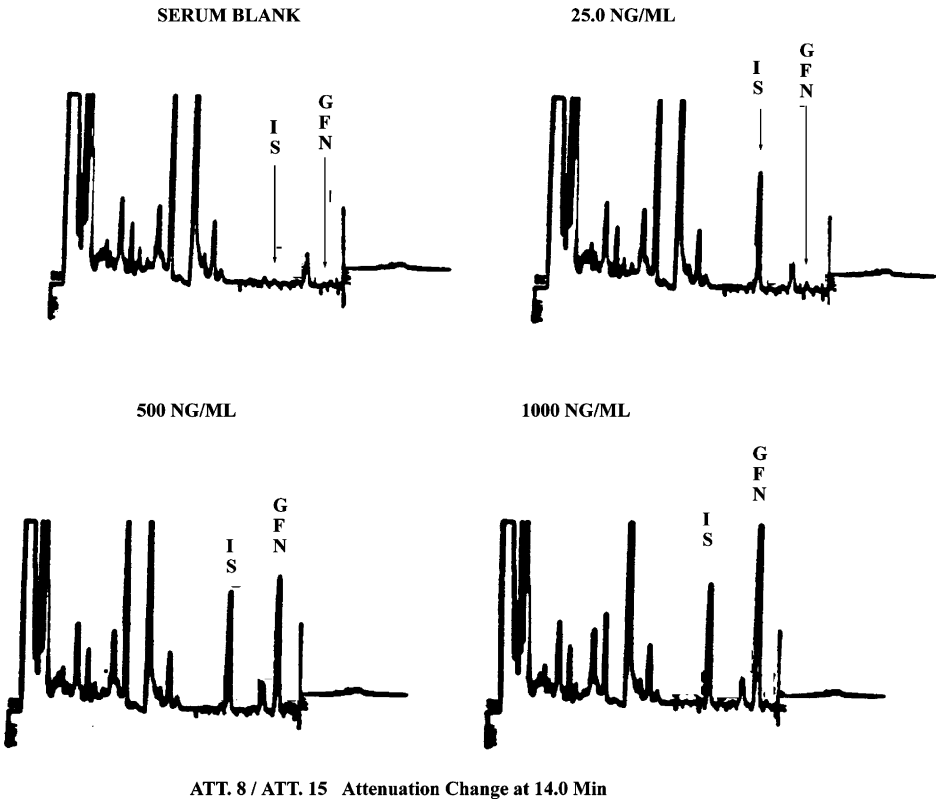

Fig. 1. Chromatograms of blank serum, 25.0, 500, and 1000 ng/ml.

3. Results

Different solvents were tried for extraction of guaifenesin from serum. *n*-Hexane/dichloromethane (1:1, v/v) gave the highest recovery, the lowest background and no interfering peaks at the retention times of guaifenesin and the internal standard and was chosen as the extraction solvent.

3.1. Method validation

All samples for the validation tests were prepared by spiking interference-free pools of serum with prepared analyte stock solutions to give the final specified concentrations.

3.1.1. Linearity

Serum samples were prepared at seven non-zero concentrations over the range of 25.0–1000 ng/ml and analyzed in duplicate. The individual natural log transformed drug/internal standard ratios (D/I) were plotted against natural log concentration. The slope, intercept, and coefficient of determination (r^2) were determined by the method of least squares (linear model). Table 1 shows the results from the linearity study. Data are presented as drug/internal standard (D/I) ratios. The ln/ln regression was characterized as having a slope of 0.872 and an intercept of −5.340 ($r^2 = 0.993$).

3.1.2. Precision and accuracy

Replicate samples ($n = 5$), prepared at three standard concentrations were used to assess intra-run precision. Selection of concentrations for analysis was made to allow for definition of precision at both extremes of the linear range. Precision is expressed as the percent coefficient of variation (%CV) for the D/I ratios (Table 2), as well as for the concentrations back-calculated from the regression analysis (Table 3). Accuracy is expressed as a percent (observed × 100/theoretical concentration).

The within-run precision of the D/I ratios ranged from 1.10 to 3.65%CV. The within-run precision of the back-calculated concentrations (using the ln/ln regression on Table 1) ranged from 1.30 to 4.20%CV. Accuracy ranged from 94.0 to 103%.

3.1.3. Multi-run validation

A more rigorous examination of both intra- and inter-run precision, accuracy as well as reproducibility,

Table 2
Precision (D/I ratio data)

	Theoretical concentration (ng/ml)		
	25.0	300	1000
Drug/internal standard ratio	0.076	0.709	1.920
	0.072	0.693	1.924
	0.073	0.692	1.963
	0.079	0.708	1.963
	0.075	0.738	1.930
Mean	0.075	0.708	1.940
S.D.	0.00274	0.0186	0.0213
%CV	3.65	2.63	1.10

Table 1
Linearity

	Theoretical concentration (ng/ml)							
	0.00	25.0	50.0	100	300	500	800	1000
Drug/internal standard ratio	0.000	0.080	0.146	0.290	0.701	1.083	1.618	1.912
	–	0.072	0.149	0.265	0.710	1.108	1.616	1.923
Mean calculated concentration (ng/ml)	0.00	23.8	50.9	105	306	507	793	964
Accuracy (%)	–	95.1	102	105	102	101	99.0	96.4
Weighted linear regression								
Slope	0.872							
Intercept	−5.340							
r^2	0.993							

Due to the slight curvilinear profile of the electron capture response, optimum linearity for this assay was obtained using ln response vs. ln concentration. Non-weighted, $1/conc^2$ weighted and polynomial regressions each proved unsatisfactory for this particular assay.

Table 3
Precision (concentration data)

	Theoretical concentration (ng/ml)		
	25.0	300	1000
Calculated concentration (ng/ml)	23.8	308	966
	22.3	300	967
	22.7	299	990
	24.9	307	990
	23.4	322	971
Mean	23.4	307	977
S.D.	0.980	9.30	12.3
%CV	4.20	3.00	1.30
Accuracy (%)	94.0	103	97.7

was assessed when the validation was transferred to a different laboratory. Control samples were prepared at three concentrations (50.0, 500, and 800 ng/ml) and quantitated in replicates of five in three separate analytical runs. Inter-run precision was calculated using the between-run variance estimate [Var(Run)] from SAS Proc Varcomp:

$$\%CV_{INTER} = \left\{ \frac{Var(Run)^{1/2}}{mean} \right\} \times 100$$

Intra-run precision was calculated similarly, using the within-run variance estimate [Var(Error)]:

$$\%CV_{INTER} = \left\{ \frac{Var(Error)]^{1/2}}{mean} \right\} \times 100$$

The inter-run precision averaged 3.93%CV (range: 2.03–7.00%CV). The intra-run precision averaged 2.25%CV (range: 1.68–3.11%CV). This latter value is comparable to that observed in the initial single-run analysis (Table 3, average %CV = 2.83). The average accuracy was 101% (compared with 98.2% in Table 3).

3.1.4. Lower limit of quantitation (LOQ)

Serum samples spiked at 0.00 (blank), 12.5, 25.0, and 50.0 ng/ml were used to assess the sensitivity of the method. Establishment of the LOQ was based upon both signal intensity and variability. Peak heights of both drug and noise (at the drug retention time in the blank) were measured manually from the chromatograms. The average signal-to-noise ratio (S/N) was then calculated at each of the concentrations. The LOQ was established as the concentration at which S/N is at least 3 and %CV (Table 2) was ≤15%.

The S/N at 12.5, 25.0, and 50.0 ng/ml was measured to be 1.5, 3, and 6, respectively. This, coupled with the precision observed in Table 3, justifies the establishment of the LOQ as 25.0 ng/ml. Example chromatograms demonstrating the relative magnitude of the signal are shown in Fig. 1.

3.1.5. Selectivity

Using 12 independent pools of serum, the selectivity of the method was examined by preparing and analyzing duplicate blanks, low standards (25.0 ng/m) and mid-high standards (800 ng/ml).

No significant chromatographic interferences were observed at the retention time of the internal standard (S/N ≥ 10) in any of the 12 serum pools tested. Nine of the 12 pools were free of significant chromatographic interferences at the retention time of guaifenesin (S/N ≥ 3).

3.1.6. Recovery

The recoveries of the drug and the internal standard were quantitated using two serum standards (50.0 and 800 ng/ml) prepared in duplicate in four serum pools (two freshly drawn). The peak heights of the processed standard samples were compared to direct injections of stock solutions prepared at concentrations, which represented approximately 100% recovery.

Recovery of guaifenesin was consistent from all pools tested, averaging 75.5% at 50.0 ng/ml with a range of 72.0–80.0 ($N = 8$ determinations) and averaging 78.0% at 800 ng/ml with a range of 76.0–81.0 ($N = 8$ determinations). The internal standard recovery was also consistent from all six pools tested, averaging 72.8% with a range of 67.0–76.0 ($N = 16$ determinations).

3.1.7. Stability

The stability of the drug was determined: (1) in processed samples, (2) through five freeze-thaw cycles, and (3) in serum stored at $-15 \pm 5\,^{\circ}C$. To establish processed sample stability, samples were injected at various times after processing. Processed stability is indicated by the consistency of the ratios injection-to-injection. The freeze-thaw and frozen stabilities were examined by quantitating the stability

of the samples against freshly prepared duplicate standard curves.

Processed samples were shown to be stable for 133 h (5 days) when stored in auto-injection vials, at room temperature, under UV-filtered fluorescent lighting.

Stability in serum through five freeze-thaw cycles ($-15 \pm 5\,^{\circ}C$ to room temperature) has been confirmed (samples, after thawing in tepid water, were allowed to stand on the bench top, under normal UV filtered fluorescent lighting, until 2 h had elapsed since their removal from the freezer).

Stability testing in frozen serum (approximately $-15\,^{\circ}C$) at three concentration levels (50.0, 800, and 1600 ng/ml) has been confirmed for 54 days.

3.1.8. Duplicate column chromatography

The linearity, sensitivity, and precision of the assay were also examined on a second similar chromatographic column. The results demonstrated consistent chromatographic results on both columns.

4. Discussion

A specific, sensitive GC assay for the determination of guaifenesin in human serum is reported. This assay is unique in that it is the first gas chromatography assay which describes the analysis of guaifenesin in a biological fluid. In addition, this assay is an improvement over all other previous gas chromatographic assays for this compound in that the sensitivity is adequate for the characterization of pharmacokinetic parameters following a single dose of guaifenesin to human subjects. Based on the procedure described herein, this assay would be amenable to laboratories which only have gas chromatographic instrumentation available. Since the procedure employes derivitization with triflouroacetic anhydride, GC/MS, with negative ion chemical ionization, would be an alternative mode of analysis following slight modification of the chromatographic conditions. To date, the assay described herein has been successfully used for the analysis of over 500 human serum samples obtained from guaifenesin dosed subjects.

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