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High-performance liquid chromatographic assay for the simultaneous determination of ipratropium bromide, fenoterol, salbutamol and terbutaline in nebulizer solution

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Abstract: A reversed-phase ion-pair high-performance liquid chromatography assay was developed for the simultaneous determination of ipratropium bromide, fenoterol hydrobromide, salbutamol sulphate and terbutaline sulphate in nebulizer solution. Chromatographic separation was achieved with a Nova-Pak C_{18} 4 μm 10 cm × 8 mm i.d. Radial-pak cartridge inside a Waters RCM 8 × 10 compression module using ternary gradient analysis. Detection was performed using UV detection at 220 nm. The standard curves were linear over the following ranges: ipratropium bromide 20.8–250.0 μg ml^{-1}, fenoterol hydrobromide 27.8–500.0 μg ml^{-1}, salbutamol sulphate 34.7–2500.0 μg ml^{-1} and terbutaline sulphate 69.5–2500 μg ml^{-1}. Inter-day and intra-day relative standard deviations for each compound ranged from 4.5–5.2% and 3.5–3.9%, respectively. The assay procedure was developed to allow the accurate determination of constituents in various combinations of nebulizer solution, as well as for stability indicating purposes. This provides a convenient means of testing long-term compatibility and stability following the post-manufacture mixing of commonly used nebulized preparations.

Keywords: *Nebulizer solutions; pharmaceutical formulations; ipratropium bromide; fenoterol hydrobromide; salbutamol sulphate; terbutaline sulphate; reversed-phase ion-pair high-performance liquid chromatography.*

Introduction

Inhalation of respirator solutions via a nebulizer is an integral component of the modern treatment of airways diseases, particularly for patients unable to use metered dose inhalers [1–3]. Nebulized drugs used in the treatment of asthma include ipratropium bromide, which is a quaternary derivative of atropine, and the β_2-agonists fenoterol, salbutamol and terbutaline. The structures of these compounds are shown in Fig. 1.

While ipratropium is not a first-line or sole drug for asthma, recent studies have advocated the use of ipratropium in acute severe asthma in combination with β_2-agonists [4–6]. In chronic obstructive pulmonary disease, various studies have shown additional clinical benefit is gained when ipratropium bromide is combined with other bronchodilating drugs [7–10]. The mixing of nebulizer solutions is a common practice [11, 12] which provides a means of shortening the time that patients spend nebulizing inhaled drugs. It has been suggested that the convenience and reduced administration time resulting from mixing nebulizer solutions may increase patient compliance [13]. The post-manufacture mixing of these nebulizer solutions, however, gives rise to issues of compatibility and stability. Iacono *et al.* [14] showed that ipratropium was stable for a period of up to 1 h after mixing with salbutamol and sodium cromoglycate, while longer term admixture stability and compatibility with fenoterol and terbutaline were not discussed. A need for a single assay that would allow testing of the compatibility and stability of different combinations of these β_2-agonists with ipratropium was evident.

HPLC has many advantages over gas chromatography for the analysis of polar, non-volatile and thermally labile compounds in aqueous matrices [15]. In this study, a reversed-phase ion-pair HPLC assay using Pic® reagent was developed to determine the concentrations of ipratropium, salbutamol, fenoterol and terbutaline present in a nebulizer solution, either alone or in any combination.

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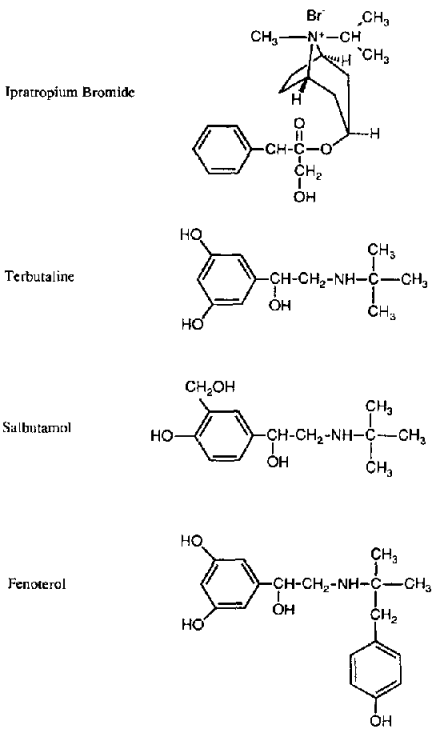

Figure 1
Structures of ipratropium bromide, terbutaline, salbutamol and fenoterol.

Ion-pair chromatography was utilized because of the quaternary structure of ipratropium; retention of the neutral ion-pair on the reversed-phase column was then possible.

Experimental

Materials

Ipratropium bromide powder was kindly donated by Boehringer Ingelheim Pty. Ltd. (Ingelheim, Germany) for the development of the assay.

The following were used in the assay procedure: ipratropium bromide (Atrovent® 0.025% nebulizer solution; Boehringer Ingelheim, Artarmon, NSW, Australia), fenoterol hydrobromide (Berotec® 0.1% w/v nebulizer solution; Boehringer Ingelheim, Artarmon, NSW, Australia), salbutamol sulphate (Ventolin® 0.5% w/v nebulizer solution; Glaxo Australia, Boronia, Vic., Australia), terbutaline sulphate (Bricanyl® 1% w/v nebulizing solution; Astra Pharmaceuticals, North Ryde, NSW, Australia), and mepivacaine hydrochloride (Carbocaine® 1% w/v sterile injection, Winthrop Laboratories, New York, USA).

Methanol and tetrahydrofuran (liquid chromatography grade) were purchased from Waters (Lane Cove, NSW, Australia). The ion pair reagent (Pic® B-8 Reagent Low UV; containing water, methanol, octane sulphonic acid and calcium acetate in undisclosed quantities) was purchased from Waters (Milford, MA, USA).

Equipment

The HPLC system consisted of a Varian Solvent Delivery System Model 9010 (Varian Chromatography Systems, Walnut Creek, CA, USA) and a Rheodyne injector Model 7161 (Rheodyne, Cotati, CA, USA) with a 10 µl external loop connected to a Varian Variable Wavelength UV–VIS Detector Model 9050 (Varian Chromatography Systems, Walnut Creek, CA, USA). Detection was performed at 220 nm.

Chromatographic peaks were digitized, integrated and recorded with a Varian GC Star Workstation (Varian Chromatography Systems, Walnut Creek, CA, USA).

Chromatographic conditions

The analysis was performed using a Nova-Pak C_{18} 4 µm 10 cm × 8 mm i.d. Radial-pak liquid chromatography cartridge (Waters, Milford, MA, USA) inside a Waters RCM 8 × 10 compression module (Waters).

Ternary gradient analysis (mobile phases A, B and C) was used to achieve satisfactory solute elution. Mobile phase A was a solution of tetrahydrofuran–distilled water (40:60, v/v) containing 0.0025 M Pic® B-8 Reagent Low UV. This was prepared by diluting the contents of one vial of Pic® B-8 Reagent Low UV in 100 ml of distilled water. Fifty millilitres of this solution was then added to 400 ml of tetrahydrofuran and made up to 1000 ml with distilled water. This resulted in a solution containing 0.0025 M Pic® B-8 Reagent Low UV which was half the recommended strength of 0.005 M (accompanying Pic® instructions). The level of ion-pair reagent was reduced to minimize the cost of the assay and did not impair performance under the conditions of this assay. Mobile phase B was distilled water and mobile phase C was methanol–distilled water (50:50, v/v). All mobile phases were filtered through a 0.5 µm filter (Lido Manufacturing, Bensenville, IL, USA) using a vacuum flask before use.

Flow rate was 2.0 ml min^{-1} of 50% mobile

phase A and 50% mobile phase B up to 7.7 min, then changing linearly to 60% mobile phase A, 15% mobile phase B and 25% mobile phase C at 13.0 min. Run time was 13.0 min with a 5.0 min equilibration time.

Calibration curve

An internal standard solution of mepivacaine was prepared by adding 2.0 ml of mepivacaine hydrochloride 1% to 8 ml of distilled water to give a solution of 2.0 mg ml^{-1}.

A 2.0 ml stock solution containing ipratropium, fenoterol, salbutamol and terbutaline nebulizer solutions (3:1:1:1, v/v/v/v) was prepared from the proprietary nebulizer solutions to give a solution containing ipratropium 125 μg ml^{-1}, fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} and terbutaline 1.67 mg ml^{-1}. Further dilutions of the stock solution were made; 200 μl aliquots of the stock solution were added to appropriate volumes of distilled water to give concentrations of 66.7, 33.3, 16.7, 8.3 and 4.2% of the stock solution concentrations.

In addition, individual solutions of fenoterol 250 μg ml^{-1}, salbutamol 2.5 mg ml^{-1}, terbutaline 2.5 mg ml^{-1} (prepared from proprietary nebulizer solutions) and ipratropium 250 μg ml^{-1} (proprietary nebulizer solution) were used to give maximum points on the calibration curves.

Sample preparation and calibration curve

Three hundred microlitres of sample solution was added to 250 μl of internal standard solution and mixed using a vortex mixer (Super-Mixer, Lab-Line Instruments, Melrose Park, IL, USA). Twenty microlitres of the mixture was injected for analysis, peak area to internal standard area ratios calculated using the Varian GC Star workstation and a calibration curve for ipratropium, fenoterol, salbutamol and terbutaline constructed by computer using a linear least squares regression method (Cricket Graph® 1.3.2, Cricket Software, Malvern, PA, USA).

Precision

Intra-day variation was calculated by analysing five individually prepared samples of 300 μl of stock solution mixed with 250 μl of internal standard solution at evenly spaced intervals throughout the sampling day. The relative standard deviation was calculated for each drug from peak/internal standard area ratios.

Inter-day variation was calculated by analysing a daily prepared sample of 300 μl of stock solution containing ipratropium 125 μg ml^{-1}, fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} and terbutaline 1.67 mg ml^{-1}, mixed with 250 μl of internal standard solution each day over a five day period. The relative standard deviation was calculated for each drug from peak/internal standard area ratios.

Stability indicating nature of the assay

Each of the four nebulizer solutions were degraded with four different conditions: heat, hydrogen peroxide, hydrochloric acid and sodium hydroxide.

Heat degradation samples were prepared by adding 1 ml of each of the proprietary nebulizer solutions to separate glass ampoules which were then sealed and heated at 200°C for 2 h. After cooling, each heat degraded sample was diluted with an appropriate volume of distilled water to give a theoretical maximum concentration equivalent to that in the stock solution (either ipratropium 125 μg ml^{-1}, fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} or terbutaline 1.67 mg ml^{-1}).

Hydrogen peroxide degradation samples were prepared by adding 100 μl of hydrogen peroxide (35% w/w) to 200 μl of nebulizer solution in a glass vial and heating at 75°C for 20 min. After cooling, each nebulizer solution was diluted with an appropriate volume of distilled water to give a theoretical maximum concentration equivalent to that in the stock solution.

Samples were degraded under acidic conditions by adding 100 μl of hydrochloric acid (2 M) to 200 μl of nebulizer solution in a glass vial and heating at 75°C for 20 min. After cooling, each nebulizer solution was neutralized with 100 μl of sodium hydroxide (2 M). Fenoterol, salbutamol and terbutaline samples were further diluted with distilled water to give final theoretical maximum concentrations equivalent to that in the stock solution.

Samples were degraded under basic conditions by adding 100 μl of sodium hydroxide (2 M) to 200 μl of nebulizer solution in a glass vial and heating at 75°C for 20 min. After cooling, each nebulizer solution was neutralized with 100 μl of hydrochloric acid (2 M). Fenoterol, salbutamol and terbutaline samples were further diluted with distilled water to give

final theoretical maximum concentrations equivalent to that in the stock solution.

An aliquot of 300 μl was taken from each degraded nebulizer solution and added to 250 μl of internal standard. All degradation samples were chromatographed under identical conditions to the calibration curve samples.

Chromatographic parameters

Chromatographic parameters of retention factor (k') and peak symmetry (A_{10}) were measured for all compounds from the calibration curve stock solution containing ipratropium 125 μg ml^{-1}, fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} and terbutaline 1.67 mg ml^{-1}.

Results and Discussion

Chromatographic conditions

The chromatogram of a stock solution sample containing ipratropium 125 μg ml^{-1} fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} and terbutaline 1.67 mg ml^{-1} with mepivacaine as internal standard is shown in Fig. 2. Retention times were as follows: salbutamol 3.2 min, terbutaline 4.3 min, ipratropium 5.9 min, mepivacaine 8.2 min and fenoterol 12.7 min. Ternary gradient analysis was used to allow the satisfactory resolution of all five compounds. A solvent with relatively strong elution power such as tetrahydrofuran was required for the satisfactory elution of the hydrophobic ion-pairs. Initially in the assay development, fenoterol was found to elute late in the chromatogram with a poor peak shape under the starting mobile phase conditions. This was corrected by two means. Firstly, mobile phase A was increased from 7.7 min to increase the proportion of tetrahydrofuran in the mobile phase, which subsequently decreased the retention time and improved the peak shape of fenoterol. Secondly, an increasing methanol com-

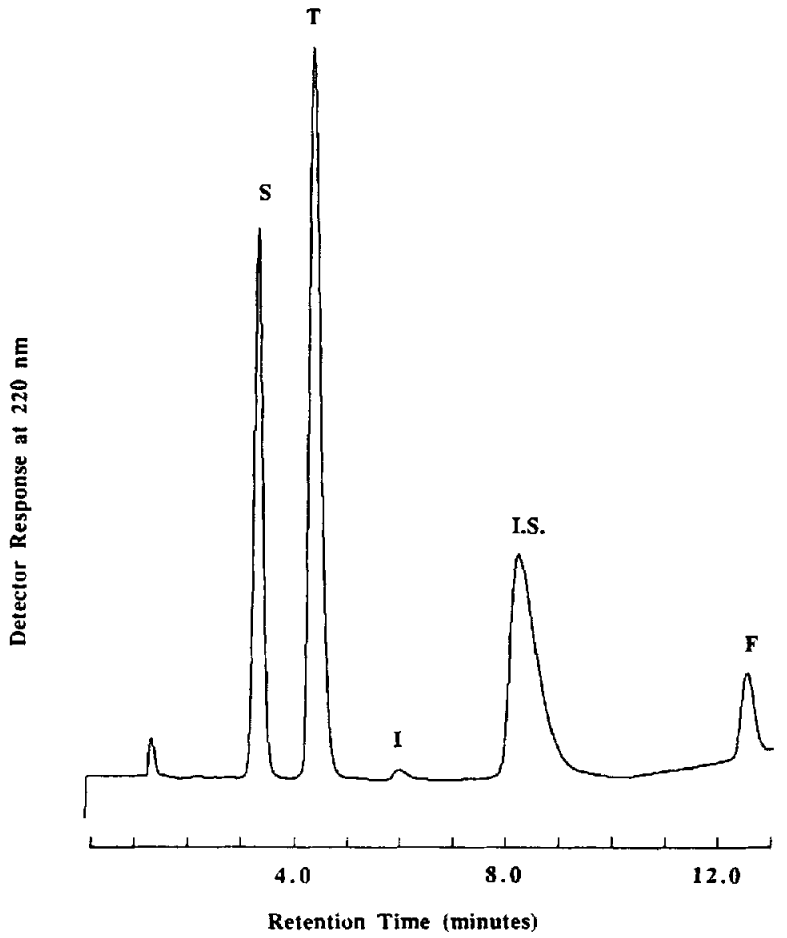

Figure 2
Chromatogram of a sample of mixed nebulizer solution containing ipratropium (I), fenotero, (F), salbutamol (S) and terbutaline (T) with mepivacaine (I.S.) as the internal standard.

ponent (mobile phase C) after 7.7 min was found to further decrease the retention time and improve the peak shape of fenoterol.

Calibration curve

The calibration curve parameters are shown in Table 1. It can be seen that the standard curves were linear over the following ranges: ipratropium 20.8–250.0 μg ml^{-1}, fenoterol 27.8–500.0 μg ml^{-1}, salbutamol 34.7–2500.0 μg ml^{-1} and terbutaline 69.5–2500 μg ml^{-1}. Detection limits for ipratropium and fenoterol were 20.8 and 27.8 μg ml^{-1}, respectively. Salbutamol and terbutaline were detectable below 34.7 and 69.5 μg ml^{-1}, respectively.

Precision

Intra-day variation was determined by calculating the relative standard deviation (RSD). The RSD for each drug was (all $n = 5$); ipratropium ±3.5%, fenoterol ±3.5%, salbutamol ±3.9%, and terbutaline ±3.9%. Inter-day variation was (all $n = 5$): ipratropium ±4.8%, fenoterol ±5.1%, salbutamol ±4.5% and terbutaline ±5.2%.

Stability indicating nature of the assay

Chromatograms for the individual solutions of heat degraded ipratropium 125 μg ml^{-1}, fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} and terbutaline 1.67 mg ml^{-1} are shown in Figs 3, 4, 5 and 6, respectively with identical attenuation. Peak areas were reduced to 3% and 54% of the salbutamol and terbutaline peak areas from the original stock solution, respectively. A higher attenuation revealed that the original ipratropium peak at 5.9 min

Table 1
Calibration curve parameters of ipratropium, fenoterol, salbutamol and terbutaline

	(n)	Linear range (μg ml^{-1})	Regression line equation (y = Peak area ratio) (x = conc. μg ml^{-1})	Correlation coefficient (r)
Ipratropium	6	20.8–250.0	$y = -0.000997 + 0.000192\,x$	1.00
Fenoterol	6	27.8–500.0	$y = 0.00251 + 0.000979\,x$	1.00
Salbutamol	8	34.7–2500.0	$y = 0.0168 + 0.000861\,x$	1.00
Terbutaline	8	69.5–2500.0	$y = 0.00392 + 0.000857\,x$	1.00

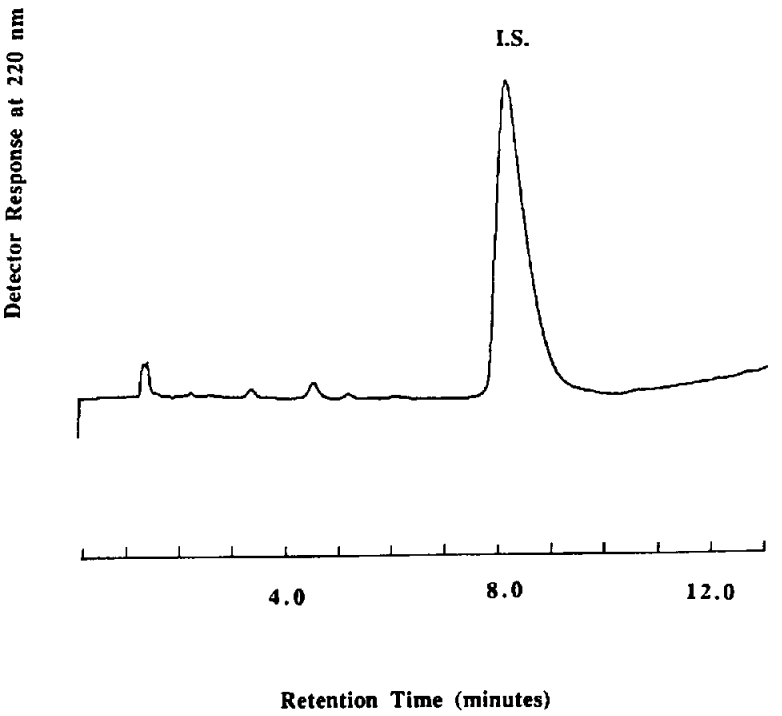

Figure 3
Chromatogram of a sample of ipratropium after heating at 200°C for 2 h with mepivacaine (I.S.) as the internal standard.

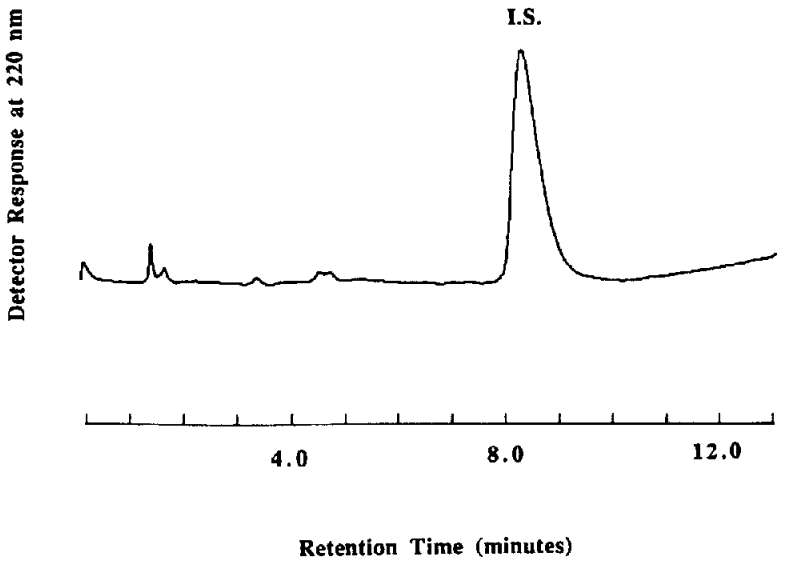

Figure 4
Chromatogram of a sample of fenoterol after heating at 200°C for 2 h with mepivacaine (I.S.) as the internal standard.

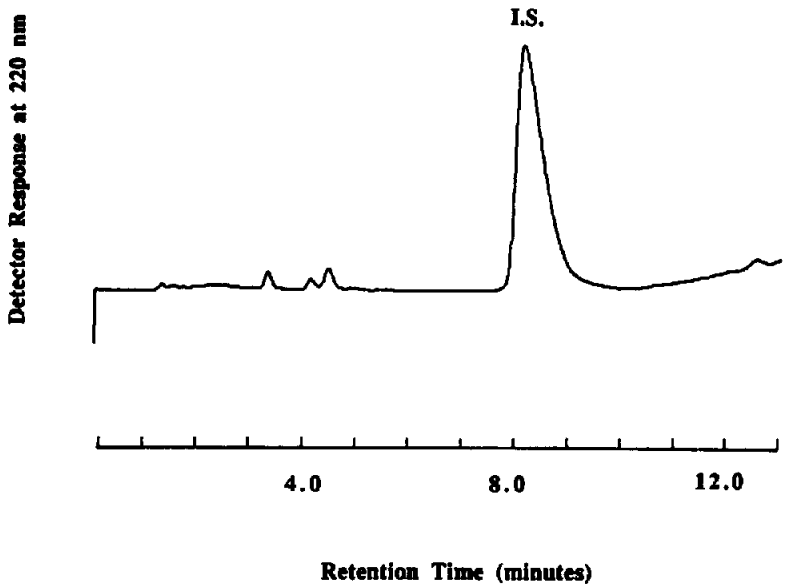

Figure 5
Chromatogram of a sample of salbutamol after heating at 200°C for 2 h with mepivacaine (I.S.) as the internal standard.

had been completely degraded with only a flat baseline remaining and a small degradation peak at 5.0 min. Similarly, the original fenoterol was completely degraded. A minor degradation product of terbutaline with a retention time of 5.6 min shouldered the non-degraded ipratropium peak (retention time 5.9 min) but did not affect the accurate integration of this peak. None of the degradation products of ipratropium, fenoterol or salbutamol produced interference peaks with the stock solution containing ipratropium, 125 μg ml^{-1}; fenoterol, 167 μg ml^{-1}; salbutamol, 833 μg ml^{-1} and terbutaline, 1.67 mg ml^{-1}.

Peak areas of samples degraded by hydrogen peroxide were reduced to 36, 71, 78 and 64% of the original ipratropium, fenoterol, salbutamol and terbutaline peak areas, respectively, with degradation product peaks in the solvent front and small peaks late in the chromatogram. Peak areas of samples degraded under acidic conditions were reduced to

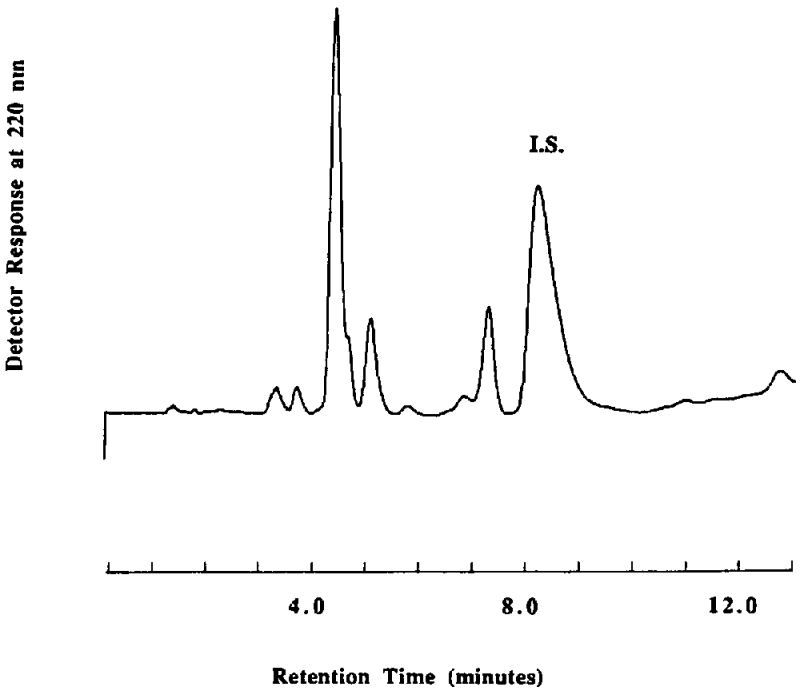

Figure 6
Chromatogram of a sample of terbutaline after heating at 200°C for 2 h with mepivacaine (I.S.) as the internal standard.

Table 2
Typical column parameters calculated from a chromatogram of a stock solution containing ipratropium 125 μg ml^{-1}, fenoterol 167 μg ml^{-1}, salbutamol 833 μg ml^{-1} and terbutaline 1.67 mg ml^{-1} with mepivacaine as internal standard

	Retention time t(min)	Capacity ratio k'	Peak symmetry (A_{10})
Salbutamol	3.2	1.6	1.13
Terbutaline	4.3	2.5	1.75
Ipratropium	5.9	3.8	2.11
Mepivacaine	8.2	5.6	2.65
Fenoterol	12.7	9.2	1.55

88, 79 and 69% of the original fenoterol, salbutamol and terbutaline peak areas, respectively, with small degradation product peaks late in the chromatogram. A lower attenuation revealed that the original ipratropium peak at 5.9 min had been completely degraded under acidic conditions with only a flat baseline remaining. Peak areas of samples degraded under basic conditions were reduced to 82, 84 and 65% of the fenoterol, salbutamol and terbutaline peak areas, respectively, with small degradation product peaks late in the chromatogram. A lower attenuation revealed that the original ipratropium peak at 5.9 min had been completely degraded under basic conditions with only a flat baseline remaining.

It was not feasible to establish mass balance for all of the drugs under all of the degradation conditions because of the many unknown decomposition products present in the samples. Furthermore, peak homogeneity could not be measured as the laboratory did not have a diode array detector. However, the assay was determined to be stability indicating for ipratropium, fenoterol, salbutamol and terbutaline under heat, hydrogen peroxide, acid and base degradation given the significant reduction in peak areas and the absence of interfering peaks from decomposition products.

Chromatographic parameters

The chromatographic parameters are shown in Table 2. There was significant peak asymmetry for mepivacaine. Peak symmetry of less than 1.5 is preferred for quantitative analysis;

however, it is generally accepted that severe peak tailing of basic drugs in reversed-phase chromatography is caused by interaction of solutes with free silanol groups in the packing [16]. Vervoot *et al.* [16] also found that the addition of an ion pair reagent (sodium 1-hexanesulphonate) did not decrease peak tailing with some protonated basic drugs, as may be the case with mepivacaine and the ion-pair reagent in this assay. The tailing of the mepivacaine peak was reduced considerably by using a relatively high proportion of tetrahydrofuran in mobile phase A. The asymmetry of this peak did not affect assay performance.

Conclusion

The assay provides a method of determining concentrations of drugs in common nebulizer solution admixtures. The assay is also stability indicating and provides a basis for assays to determine the stability of common nebulizer admixtures containing ipratropium and β_2-agonists. Work on these stability studies has commenced.

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