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Optimization of a column liquid chromatographic procedure for the determination of plasma salbutamol concentration

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Abstract

A reversed-phase column liquid chromatographic procedure with fluorescence detection for the determination of salbutamol in plasma is described. A 1-ml aliquot of the sample, after the addition of bamethan as the internal standard, is passed through a Bond Elut silica extraction column. The column is selectively washed to remove neutral, acidic, and weakly basic compounds. The desired compounds are eluted with a 1-ml aliquot of methanol. The eluate is evaporated under vacuum at ambient temperature and the residue is reconstituted in 40 μl of the mobile phase which contains octanesulfonic acid as the ion-pairing reagent. The entire extract is injected onto a 150 × 4.6 mm I.D. column packed with 5-μm octylsilica particles. Peaks are detected with a fluorescence detector (excitation wavelength = 275 nm, emission wavelength = 310 nm). In the resulting chromatogram, salbutamol and the internal standard give sharp peaks that are well resolved from the extraneous peaks. The procedure allows the quantitation of salbutamol down to 0.2 ng/ml.

1. Introduction

Salbutamol is still the drug of choice for the treatment of acute episodes of asthma and other obstructive airway diseases. It has now been accepted that the optimal route to administer a bronchodilator is by a metered dose inhaler (MDI). An MDI rapidly supplies an effective concentration of the drug as an aerosol to the airways directly, with the least side effects [1]. Salbutamol is also delivered by MDI to patients receiving mechanical ventilation. A number of different MDI devices can be integrated into the ventilator circuit to deliver the doses. The efficiency of these different aerosol devices can be determined by comparing the resulting plasma salbutamol concentrations at a given time after the delivery of a fixed number of doses of the drug.

Column liquid chromatography (LC) is to date the most practical technique for the determination of drugs in biological matrices. A large number of LC procedures [2–15] have been described for the determination of salbutamol in plasma or urine. Recently, another LC procedure has been described for the determination of salbutamol in rat tissue [16]. However, the detection limit of most of these procedures is not adequate for the required purpose. It was the objective of this investigation to select conditions

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for an LC procedure which allow quantitation of salbutamol at 0.2 ng/ml of plasma. Reagents for immunoassays [17,18] for salbutamol are not available commercially and show high cross reactivity with drug metabolites.

The method described below has been developed for use in the following research study. Patients in the Intensive Care Unit of St. Joseph's Hospital receiving mechanical ventilation are being recruited to receive salbutamol aerosol by MDI (Ventolin; Glaxo, Toronto, Canada) containing technetium pertechnetate as a tracer for the drug in the formulation. Delivery of the drug to the lungs will be monitored by a bedside mobile gamma camera and blood samples collected after the delivery of 4, 20 and 36 doses of the drug (100 μg/dose). Plasma separated from these samples will be stored frozen at −80°C and analyzed for salbutamol concentration when the required number of patients has been recruited. The salbutamol levels measured will be correlated with the deposition of the drug in the lung.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Deionized water was distilled in an all-glass still.

Stock salbutamol solution (1.0 g/l). A 10-mg amount of salbutamol (Sigma, St. Louis, MO, USA) was dissolved in 10 ml of methanol. This solution was stored at −20°C. The solution was stable for at least 6 months.

Working salbutamol solution (1.0 mg/l). A 25-μl aliquot of stock salbutamol solution was diluted to 25 ml with 0.01 *M* phosphoric acid.

Plasma salbutamol standards. Aliquots of 50 and 100 μl of working salbutamol solution were diluted to 50 ml with pooled plasma in volumetric flasks to give 1 ng/ml and 2 ng/ml salbutamol standards respectively. The standards were stored frozen at −80°C in 2-ml aliquots. They were stable for at least four months.

Stock internal standard solution (1 g/l). A 12.3-mg amount of bamethan sulfate (Sigma) was dissolved in 10 ml of methanol. The solution was stored at −20°C and was stable for at least 6 months.

Intermediate internal standard solution (10 mg/l). A 250-μl aliquot of the stock internal standard solution was diluted to 25 ml with water. The solution was stored at 4°C and prepared every month.

Workinq internal standard solution (50 μg/l). A 50-μl aliquot of the intermediate internal standard solution was diluted to 10 ml with 1% potassium bicarbonate solution. This solution was prepared when required.

2.2. Sample collection

The whole blood samples from patients in the Intensive Care Unit at St. Joseph's Hospital are collected in 7-ml Vacutainer tubes containing EDTA (Becton Dickinson, Rutherford, NJ, USA). Samples are obtained pre- and post-treatment with 4, 20 and 36 doses of inhaled salbutamol (100 μg/dose). The plasma is separated within 1–2 h of sample collection and stored at −80°C till analysis.

2.3. Extraction

The required number of l-ml Bond Elut silica columns (Varian, Harbor City, CA, USA) was placed on a VacElut system. The columns were washed twice at a slow rate with l-ml aliquots of methanol and once with water, each time aspirating the liquid completely using mild suction. The suction was stopped completely and the system vacuum released. An aliquot of 0.2 ml of the working internal standard solution and 1 ml of the sample (standard or unknown) were applied to each column. After *ca.* 2 min, mild suction was applied so that the samples passed through the columns at a slow rate of less than 1 ml/min. The suction was increased after the samples had passed through the columns, to expel all the trapped liquid. The columns were washed at a slow rate twice with 1-ml aliquots of water and once with a l-ml aliquot of acetonitrile each time increasing the suction for a few seconds to drain the columns completely. Dispos-

able glass tubes (12 × 75 mm) were washed with water and methanol, dried, labelled and placed in 16 × 100 mm glass tubes. The columns were placed on correspondingly labelled tubes and eluted once with 1-ml aliquots of methanol by gravity. The last drop of methanol was pushed out of the column by applying pressure with a Pasteur pipette fitted with a large squeeze bulb. The eluate was evaporated in a vacuum evaporator without switching on the heater (Savant Instruments, Farmingdale, NY, USA). The residue was dissolved in 40 μl of the mobile phase. The tubes were vortex-mixed and centrifuged for 20 s at 300 *g*. The entire solution was injected.

2.4. Chromatography

A modular chromatographic system consisting of a Model LC-7A pump (Shimadzu, Columbia, MD, USA), a 50-μl loop injector (Model 7125, Rheodyne, Cotati, CA, USA), a reversed-phase 150 × 4.6 mm I.D. column packed with Ultrasphere Octylsilica (5 μm particles, Beckman, Berkley, CA, USA) protected by a 15 × 3.2 mm I.D. C_8 guard cartridge (7 μm particles, Brownlee Labs, Santa Clara, CA, USA) and a Model RF 535 fluorescence detector (Shimadzu), was used.

Chromatography was performed at ambient temperature and the peaks were recorded with a plotter integrator (Model C-R 501, Shimadzu). A mobile phase (900 ml of water + 150 ml of methanol + 50 ml of acetonitrile + 0.25 ml of phosphoric acid + 750 mg of potassium dihydrogen phosphate + 50 mg of octanesulfonic acid) was used at a flow-rate of 1.6 ml/min with an operating pressure of 15 MPa (2175 psi).

3. Results and discussion

3.1. Choice of internal standard

Use of an internal standard is helpful in the processing of biological samples for the precise determination of low concentrations of drugs. Hutchings *et al.* [3], Ong *et al.* [9], and Sagar *et al.* [14] did not use any internal standard in their determination of salbutamol. Kurosawa *et al.* [4] used ethnizamide, Tan and Soldin [5] and Tamisier-Karolak *et al.* [11] used fenoterol, and McCarthy *et al.* [15] used atenolol, as the internal standards for the assay of salbutamol. These compounds are less than ideal as internal standards for the salbutamol assay as they are significantly different in their structures from that of salbutamol. Fenoterol, having two phenolic groups, is highly electroactive compared to salbutamol which has only one phenolic group [2]. Pholedrine used by Jarvie *et al.* [7], and bamethan, used by a number of investigators [2,6,8,10,12,13] as the internal standard for salbutamol assay, have structures similar to that of salbutamol. We selected bamethan as the internal standard because it is readily available commercially.

3.2. Detection

Salbutamol is weakly electroactive and has fair native fluorescence. Consequently, both electrochemical and fluorescence detection have been successfully used for the LC determination of salbutamol. We have compared the two modes of detection to select the technique which allows the maximum sensitivity. For electrochemical detection, a Model 5100 coulochem detector equipped with a high sensitivity (No. 5011) analytical cell and a (No. 5020) guard cell (ESA, Bedford, MA, USA) was used. The guard cell, set at +0.9 V and placed between the pump and the injector, reduces the background noise by oxidising the impurities of the mobile phase. Electrode I of the analytical cell was set at +0.5 V and electrode II of the analytical cell was set at +0.8 V. The signal of electrode II was monitored (output = 1 V, gain = 1 × 20, integrator attenuation = 3). These settings are similar to those reported by Emm *et al.* [8].

Salbutamol has been detected fluorometrically at different settings of excitation and emission wavelengths. These settings can be divided into 3 groups; *i.e.* ex = 273–276 nm, em = 309–310 nm [4]; ex = 220–230 nm, em = 307–310 nm [3,6]; and ex = 200–225 nm with no emission filter

[9,10,13,15]. The detector used in this study showed a very poor response for salbutamol when excitation was set at 225 nm and a zero order emission was set. The instrument has two monochromators and there is no provision to remove or insert an emission filter. The response of salbutamol was *ca.* 40% higher at ex = 225 nm and em = 310 nm than that at ex = 275 nm and em = 310 nm. However, with the aging of the lamp, the baseline at ex = 225 nm became quite noisy. As a result, ex = 275 nm and em = 310 nm were considered optimal for the detection of salbutamol. Hindle and Chrystyn [12] used 276 nm as the excitation wavelength and 609 nm as the emission wavelength. At this emission wavelength, baseline noise decreased by *ca.* 30% but the response of salbutamol reduced to half of that observed at 310 nm. Fig. 1A shows the response and baseline noise when 1 ng of salbutamol and 10 ng of bamethan are injected on the column.

The coulochem detector under the above mentioned conditions showed salbutamol response similar to that showed by the fluorescence detector. However, operation of the coulochem detector was quite tedious as it took too long a time to stabilize the baseline after the start-up or after changing the flow-rate of the pump. Furthermore, in some instances, the solvent peak after the injection of a plasma extract was too large and did not return to baseline prior to the elution of salbutamol.

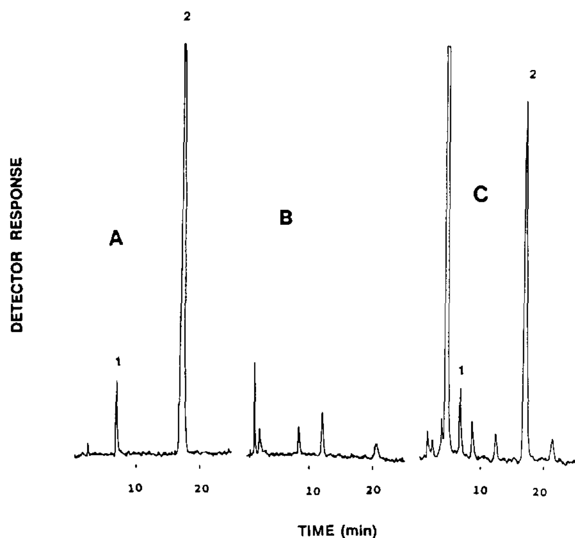

Fig. 1. Chromatograms of (A) 40 μl injection of an unextracted standard of 25 ng/ml of salbutamol + 250 ng/ml of bamethan, (B) of the entire extract of 1-ml aliquot of drug free plasma, and (C) of the entire extract of 1-ml aliquot of plasma of a patient after receiving four puffs of salbutamol. Peak 1 = salbutamol, peak 2 = bamethan. Unlabelled peaks are extraneous.

3.3. Extraction

A number of diverse extraction techniques have been used to isolate salbutamol from biological matrices. Hutchings *et al.* [3] and Miller and Greenblatt [6] used di-(2-ethylhexyl)phosphate as an ion-pairing reagent for liquid–liquid extraction of salbutamol. Tan and Soldin [5] applied this ion-pairing extraction after prepurification of plasma samples with Sep-Pak C_{18} cartridges. We avoided the use of ion-pairing extraction as it required the use of water immiscible solvents and meticulously clean and deactivated glassware. Oosterhuis and Boxtel [2] and Tamisier-Karolak *et al.* [11] performed a preliminary clean-up with C_{18} extraction columns and achieved further clean-up of extracts by online switching cation-exchange and CN-silica precolumns respectively. Sagar *et al.* [14] applied the plasma sample directly to a C_{18}-silica loop column. We could not evaluate this approach because of lack of the additional equipment required for the automated switching of the loop columns. Ong *et al.* [9] report a clean chromatogram of plasma extracts obtained after immunoaffinity chromatography. However, we were not able to use this technique as the antibody for salbutamol is not commercially available. Further, this technique does not allow the use of an internal standard, which we regard as a limitation of the method.

Extraction columns packed with C_{18}-silica are commonly used for solid-phase extraction of drugs including salbutamol [2,4,5,8,11,13]. However, we could not optimize the extraction of salbutamol using C_{18} extraction columns as has been observed by Bland *et al.* [10] and by McCarthy *et al.* [15]. There is an increasing trend

to use cation-exchange (SCX) [20] or mixed-mode, containing cation-exchange and hydrophobic groups [21–23], extraction columns for the isolation of basic drugs. We evaluated Bond Elut 1-ml SCX (Varian, Harbor City, CA, USA) and 1-ml CleanScreen DAU (Worldwide Monitoring, Horsham, PA, USA) columns for the extraction of salbutamol from plasma. The traditional approach to condition these extraction columns, by washing with methanol, gave large extraneous peaks when only deionized water was used as the sample. These extraneous peaks were reduced when the columns were initially washed three times with 5% ammoniacal methanol, twice with elution reagent (dichloromethane–isopropanol–ammonium hydroxide, 75:25:2, v/v), once with methanol and with water and buffer. The extraneous peaks were further reduced when the columns were reused for the third and fourth time. The recovery of salbutamol and the internal standard was greater than 90% and was unaffected by the reuse of the columns up to 5 times. The extraction with these columns became convenient when the supernatant obtained by treating 1 ml of plasma sample with 250 μl of 10% perchloric acid was processed. However, the recovery of the drug dropped to *ca.* 80%. With this extraction approach good linearity and reproducibility were obtained over the 0.5–10 ng/ml range tested (within-batch C.V. of 5.2% for a spiked sample of 5 ng/ml, $n = 10$). The procedure allowed the desired detection limit of 0.2 ng/ml. However, conditioning of these columns was quite tedious. Recently, another type of column for solid-phase extraction has been introduced by TOXI-LAB (Irvine, CA, USA). Each of these columns have only 15 mg of sorbent as compared to 100 mg of sorbent in the traditional column. The sorbent in these new columns has been compressed into a rigid disc which requires reduced amounts of solvent for washing the impurities and elution of analytes. A VC-MP 3 extraction column consisting of mixed-mode sorbent gave reduced background peaks after simple activation of the column with methanol compared with Bond Elut SCX or CleanScreen DAU columns. However, these extraction columns could not be evaluated further as serum/plasma samples passed through the extraction discs with great difficulty.

We also evaluated silica extraction columns as suggested by Bland *et al.* [10] and McCarthy *et al.* [15]. Use of silica extraction columns have not been popular for the isolation of drugs from aqueous matrices as the manufacturers of solid-phase extraction columns have recommended that Si columns be used with non-aqueous matrices. With the silica columns, good recovery in the range of 85–94% of salbutamol and bamethan was achieved with relatively clean extracts. Unlike SCX columns, only few extraneous peaks were observed when de-ionized water was processed through a silica extraction column. We have confirmed the observation of McCarthy *et al.* [15] that the sample and the internal standard can be applied separately to the extraction column directly rather than as a pre-mixed mixture as suggested by Bland *et al.* [10], without any decrease in the recovery of the drug or the internal standard or any adverse effect on the precision. We have also observed an improvement of the precision when the pH of the sample was adjusted to *ca.* 9 and that 1 ml of methanol rather than 2 ml is adequate to completely elute the adsorbed drugs from the silica column. It appears that McCarthy *et al.* [15] washed the columns with water only after application of the sample. We have confirmed the observation of Bland *et al.* [10] that the columns after the application of the sample can be washed with water and 1 ml of acetonitrile without any loss of salbutamol and bamethan. However, the recovery of salbutamol was poor when plasma proteins were precipitated with 1 volume of acetonitrile and the resulting supernatant was applied to the extraction column. Extraction of salbutamol with a silica column is fairly selective as acids (*e.g.* salicylic acid), acidic drugs (*e.g.* acetaminophen) and neutral or weakly basic drugs (*e.g.* diazepam) are not recovered to any significant degree. The recovery of salbutamol and bamethan was quite consistent within a batch and between batches of extraction. However, the recovery of extraneous peaks was highly variable even when samples of the same plasma pool were processed. In a majority

of cases, there was no significant extraneous peak after the elution of bamethan. However, to avoid any interference of the late eluting peak with the salbutamol peak, injections were made after every 25 min. As suggested by McCarthy *et al.* [15] we reused the extraction columns three times without adversely affecting the recovery of drugs or the cleanliness of the extracts.

3.4. Chromatography

Conditions for chromatographic separation were selected to meet the twin objectives of high sensitivity and selectivity. At the required sensitivity, it was not possible to eliminate all the plasma extraneous peaks by a one-step extraction procedure. The separation of salbutamol and the internal standard from these extraneous peaks has to be achieved by optimal chromatographic conditions. We selected catecholamines as model compounds to optimize the chromatographic conditions as catecholamines are present in plasma at low concentrations and have the same functional groups as salbutamol. Ion-pairing reversed-phase liquid chromatography has been the most commonly used approach for the determination of plasma catecholamines [24]. Indeed, ion-pairing reversed-phase LC has been used for the determination of salbutamol by both electrochemical [5,7,14] and by fluorescence [4,12] detection. Reversed-phase ODS columns have been used by a majority of the investigators for the determination of salbutamol. However, Kurosawa *et al.* [4] used an Octyl column and Oosterhuis and Boxtel [2] used a RP-2 column. We observed sharp and well separated peaks of salbutamol and bamethan with the use of an ODS and an Octyl column. However, only the Octyl column allowed optimal separation of the extraneous peaks from the desired peaks (Fig. 1B and C) with the described mobile phase. The separation of the salbutamol peak from the extraneous peak was lost when octanesulfonate was replaced by perchlorate as the counter ion. McCarthy *et al.* [15] have pointed out that the use of a cation-exchange analytical column was essential to separate salbutamol from terbutaline. Our system also shows only minimal separation of salbutamol and terbutaline. However, Sagar *et al.* [14] reported the separation of salbutamol and terbutaline with the use of an ODS column and dodecyl sulphate as the counter ion.

3.5. Method validation

The relation between the ratios of peak areas of salbutamol/internal standard was linear for the range tested (0.6 to 20 ng/ml) and the curve passes through the origin ($y = 0.004 + 0.081\times$, $r^2 = 0.999$). Low noise of the baseline, good extraction recovery and relatively high detector response allow the quantitation of salbutamol down to 0.2 ng/ml. Analysis of plasma spiked with salbutamol at high concentrations showed a within-batch C.V. of 2.7% (mean = 9.2 ng/ml) and between-batch C.V. of 5.6% (mean = 9.2 ng/ml). Analysis of plasma spiked with low concentrations of salbutamol showed a within-batch C.V. of 14.7% (mean = 0.3 ng/ml) and a between-batch C.V. of 16.6% (mean = 0.3 ng/ml). In all cases $n = 10$. Drug free plasma spiked with a nominal concentration of salbutamol of 1.2 ng/ml when analyzed in duplicate gave an average value of 1.1 ng/ml. A negative bias of *ca.* 8.4% between the observed and the spiked values indicate that the procedure is fairly accurate.

To check for the possible interference of endogenous compounds arising from different diets, non-fasting samples from 20 volunteers were collected. Some of the volunteers were taking analgesics, antihistamines, or contraceptives. Plasma obtained from these samples was analyzed without the addition of the internal standard. No peak which could interfere with salbutamol or bamethan was observed in any of these samples. In a preliminary experiment, blood was collected from a patient receiving mechanical ventilation prior to and 10 minutes after the delivery of four puffs each of 100 μg of salbutamol (Ventolin, Glaxo). The baseline sample was negative for salbutamol and the sample after the administration of the drug showed a salbutamol concentration of 1 ng/ml (Fig. 1C). The above described methodology appears to be

a suitable procedure for the determination of salbutamol in plasma following an inhaled therapeutic dose of the drug.

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