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Short communication

LC–MS method for the determination of albuterol enantiomers in human plasma using manual solid-phase extraction and a non-deuterated internal standard

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Abstract

A sensitive enantioselective liquid chromatography–mass spectrometry (LC–MS) assay using a manual solid-phase extraction (SPE) procedure, a non-deuterated internal standard and an ion trap LC–MS was developed to measure (*R*)- and (*S*)-albuterol in plasma. Sample extraction from plasma was achieved by a manual SPE extraction procedure with methoxyphenamine added as the internal standard. Chiral separation was achieved using a teicoplanin-based stationary phase and a mobile phase consisting of methanol, acetic acid and 28% (w/v) ammonia (1000:5:1, v/v/v). Samples were analyzed by selected reaction monitoring of product ions from the protonated molecular ions. The detection limit of the assay was 0.1 ng/ml with a conservative lower limit of quantification of 0.25 ng/ml for each enantiomer. Recovery of albuterol enantiomers from plasma spiked at 10 ng/ml of racemate was determined to be 89 ± 5.8% (mean ± S.D.). Reproducibility at 10 ng/ml of racemate assessed by the coefficient of variation was found to be 6.5% ($n = 5$). Instrument precision (measured as coefficient of variation) was 1.4% ($n = 5$). The correlation coefficient r^2 determined from the calibration curve over the range 0.5–50.0 ng/ml racemate in plasma was 0.998. This assay allows adequate sensitivity, recovery and reproducibility for the application to studies of inhaled albuterol.

Keywords: Albuterol; Chiral; Assay; Enantiomer; Liquid chromatography–mass spectrometry

1. Introduction

Albuterol (salbutamol) is a short-acting β_2-agonist used to relieve bronchoconstriction in asthma (Fig. 1). Until recently, albuterol has been administered as a racemic mixture (*R*,*S*)-albuterol; however, only the (*R*)-albuterol enantiomer is considered to be pharmacologically active. The inactive (*S*)-albuterol enantiomer has, however, been associated with increased airway responsiveness to provocation in vitro and in guinea pigs [1,2]. This effect has not been shown in human, where the evidence of adverse effects

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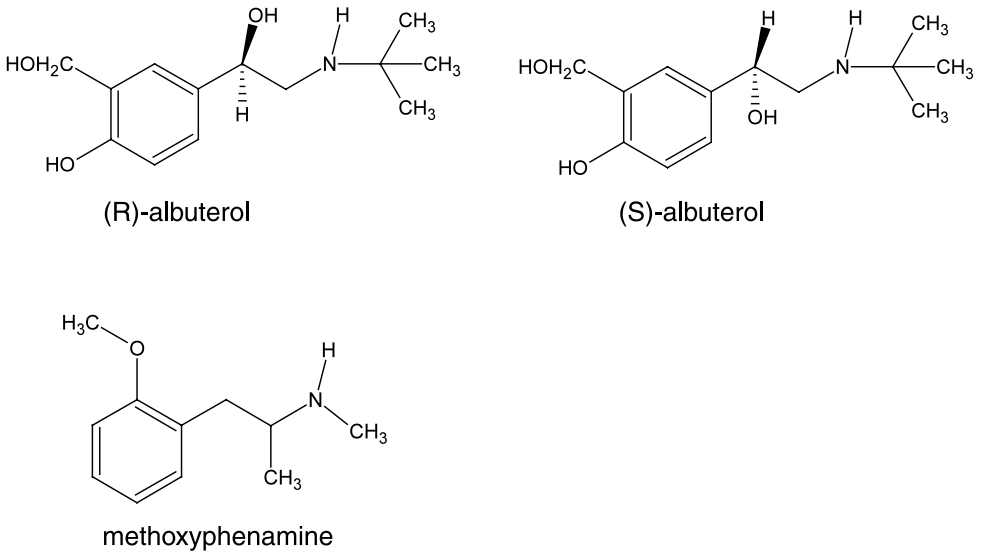

Fig. 1. Structures of albuterol enantiomers and methoxyphenamine internal standard.

attributed to the (*S*)-albuterol enantiomer is less clear-cut and is the subject of much debate [3–5]. There is also some evidence to suggest that (*S*)-albuterol acts as a functional antagonist to active (*R*)-albuterol [6]. Adding to the controversy is the fact that (*R*)- and (*S*)-albuterol possess different pharmacokinetic profiles leading to the accumulation of drug plasma levels [7–11].

The need to elucidate the role of *S*-albuterol pharmacokinetics and pharmacodynamics in asthma with inhaled therapy has resulted in a need for a chiral assay with the sensitivity to measure low plasma levels. Albuterol is most commonly administered by the inhalation route with peak plasma levels of the racemate around 5 ng/ml after a dose of between 800 and 1200 μg racemic albuterol [7,8].

There have been several recent assays reported in the literature that have been used for the determination of plasma levels of albuterol enantiomers after the administration of the drug via the inhalation route. The methods employed in these assays have included HPLC with fluorescence detection (LLoQ of 0.25 ng/ml and detection limit of 0.10 ng/ml, respectively) [7,8], HPLC with electrochemical detection (LLoQ of 0.25 ng/ml) [11], liquid chromatography–mass spectrometry (LC–MS; LLoQ of 0.025 ng/ml) [12] and GC–MS (LLoQ of 0.05 ng/ml) [13].

CE has been reported [14], but the LLoQ of 0.5 μg/ml has been insufficient for studies involving administration by the inhalation route. Given the drawbacks of extensive sample preparation, derivatization steps and long analysis times associated with GC–MS analysis, the study by Joyce et al. [12] which employed LC–MS detection is arguably the gold standard for plasma albuterol enantiomer determinations.

There are, however, some disadvantages with this assay [12] such as the cost of the apparatus and instrumentation and the availability of a suitable internal standard. Joyce et al. [12] employed a deuterated albuterol internal standard, a custom manufactured robotic solid-phase extraction (SPE) system and triple quadrupole LC–MS instrumentation which were unavailable in our laboratory. The method reported here is adapted from that of Joyce et al. to use a non-deuterated internal standard, a manual SPE technique and an ion trap LC–MS instrument allowing the assay to be performed in a more routine laboratory environment. Assay sensitivity, accuracy, recovery and

reproducibility are examined and the method is applied to a plasma sample from an asthmatic patient administered albuterol.

2. Experimental

2.1. Chemicals

Racemic albuterol pure base was purchased from Sigma Aldrich Pty. Ltd. (New South Wales, Australia) and methoxyphenamine hydrochloride USP from Upjohn Pty. Ltd. (New South Wales, Australia). Other reagents included HPLC grade methanol (Hipersolv, BDH-Merck, Crown Scientific, New South Wales, Australia) and analytical reagent grade ammonia solution 28% (Ajax Chemicals, APS, New South Wales, Australia).

2.2. LC–MS system

A teicoplanin-based ASTEC Chirobiotic T column (250×4.6 mm ID) and matching guard column were purchased from Alltech Associates Australia Pty. Ltd. (Victoria, Australia) and was used for its chiral separation properties. The albuterol enantiomers were analyzed using a Waters Alliance 2690 HPLC coupled to a Finigan LCQ mass spectrometer fitted with an atmospheric pressure chemical ionization (APCI) source.

The HPLC was operated isocratically with a mobile phase of methanol, acetic acid and 28% (w/v) ammonia (1000:5:1, v/v/v) at an initial flow rate of 2.0 ml/min and then at 1.3 ml/min after 2 min. The APCI conditions were nitrogen sheath gas 50 psi, source current 6 μA, capillary temperature 150 °C, and vaporizer temperature 400 °C. Samples were analyzed by selected reaction monitoring of product ions from the protonated molecular ions; for albuterol, the fragmentations *m*/*z* 240_*m*/*z* 222 and *m*/*z* 240_*m*/*z* 166 were monitored, while for methoxyphenamine the fragmentation monitored was *m*/*z* 180_*m*/*z* 149. Collision energy was optimized at 17% for albuterol for the *m*/*z* 222 ion, with an isolation width of 3 Da.

2.3. Solid-phase extraction

Plasma samples were extracted with Waters Oasis™ 30 mg (1 ml) SPE HLB cartridges (Waters Australia Pty. Ltd., New South Wales, Australia). The extraction procedure utilized a Vac-Elut™ (Analytichem International; Alltech Australia, Pty. Ltd., Victoria, Australia) and allowed extraction of 10 cartridges in a batch. A vacuum of approximately 15 mmHg was continuously regulated during the procedure to obtain a near constant flow through the cartridges and at no stage were the cartridges allowed to dry.

The cartridges were conditioned with 500 μl of methanol, equilibrated with 500 μl of water, then loaded with a mixture of 500 μl of ammonia buffer (pH10) and 500 μl of plasma sample. The SPE cartridges were then washed with 500 μl of water before they were removed from the Vac-Elut™ and the albuterol eluted into tapered glass test tubes with four consecutive 250 μl washes of methanol (total 1 ml) under positive pressure using a 6 ml disposable syringe. Fifty microliters of the internal standard solution (containing methoxyphenamine hydrochloride 50 μg/100 ml of water) was added and the eluent containing internal standard was taken to dryness under a stream of heated nitrogen (45 °C). The extracts were then reconstituted in 100 μl of methanol, mixed using a vortex mixer (Super-mixer, Lab-line Instruments, Inc., IL), transferred to mass spectroscopy sample vials and stored at 4 °C prior to analysis by LC–MS/MS.

2.4. Calibration

Primary stock solutions of racemic albuterol (1 mg/ml) in methanol were prepared to spike plasma samples and estimate recovery of albuterol from the SPE procedure. Secondary solutions of 0.01, 0.1 and 1 μg/ml of albuterol in water were freshly prepared 24 h prior to use and were used to spike blank human plasma (obtained from a volunteer with no history of albuterol use) to produce calibration standards at 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 ng/ml of racemic albuterol. These calibration levels were equivalent to 0.25, 0.5, 2.5, 5.0, 12.5 and 25 ng/ml of (*R*)- and (*S*)-albuterol.

2.5. Assay validation

The sensitivity of the assay was examined by determining the detection limit (based on a signal to noise ratio of 3) and the LLoQ estimated from the lowest concentration with a coefficient of variation of less than 15%. An estimate of recovery was estimated by comparing the peak areas of albuterol from plasma samples ($n = 5$) spiked at 10 ng/ml of racemate compared with a direct injection of aliquots ($n = 5$) from a 50 ng/ml albuterol solution in water (equivalent to 100% theoretical recovery after a 5-fold concentration in the SPE process). Intra-day reproducibility of the assay was assessed by determining the coefficient of variation for five replicate blank plasma samples spiked with racemic albuterol at 10 ng/ml (equivalent to both (*S*)- and (*R*)-albuterol at 5 ng/ml). The instrument precision was examined by injecting multiple injections of the same 10 ng/ml sample five times. The correlation coefficient was determined from the calibration curve over the range 0.5–50.0 ng/ml racemic salbutamol in plasma. Intra-assay accuracy was expressed as percentage error ([found concentration − spiked concentration]/spiked concentration × 100) at the 10 ng/ml plasma level. The selectivity factor α describing the separation of the enantiomers was calculated.

2.6. Application of the assay in an acute asthmatic patient

A plasma sample was obtained from a patient presenting to the local emergency department with acute asthma. Informed consent was given by the patient, the blood sample collected, centrifuged, the plasma harvested and stored at −20 °C until analysis. This study was approved by the Southern Tasmania Health and Medical Human Research Ethics Committee.

3. Results

The detection limit of the assay was determined as 0.1 ng/ml with a conservative LLoQ of 0.25 ng/ml for each enantiomer. Recovery of albuterol enantiomers from spiked plasma at 10 ng/ml of racemate was determined to be 89 ± 5.8% (mean ± S.D.). Reproducibility at 10 ng/ml of racemate assessed by the coefficient of variation was found to be 6.5% ($n = 5$). Instrument precision (measured as coefficient of variation) was 1.4%. The correlation coefficient r^2 determined from the calibration curve over the range 0.5–50.0 ng/ml in plasma was 0.998. Intra-assay accuracy was found to be −10.2% at the 10 ng/ml plasma level. The selectivity factor α describing the separation of the enantiomers was 1.15. Differences in LLoQ, and recovery and reproducibility between enantiomers were not observed.

A plasma sample obtained from a patient presenting to the local emergency department with acute asthma is shown in Fig. 2 with near baseline separation under the chromatography conditions described, shown along with the methoxyphenamine internal standard. The retention times of (*R*)- and (*S*)-albuterol were 4.1 and 4.5 min, respectively, the retention time for methoxyphenamine was 5.7 min and the run time 7.0 min. The MS–MS product ion spectrum from the protonated albuterol molecular is shown in Fig. 3. While the m/z 166 as used by Joyce et al. [13] on a triple quadrupole MS provides more selectivity, its sensitivity on the ion trap was well below that of the m/z 222 ion at all collision energies; it was therefore only included as a confirmation ion.

4. Discussion

The SPE and analytical methods described by Joyce et al. [12] for chiral albuterol determinations in plasma are limited by the cost of the apparatus and instrumentation, and availability of a suitable internal standard. This paper describes a modified assay and its application, adapted from the work by Joyce et al. [12], that can be undertaken in an analytical laboratory with an ion trap LC–MS instrumentation, with no specialized robotic SPE apparatus or suitable deuterated albuterol internal standard.

Initially external standardization by using a fixed loop LC injection was investigated as a means of determination; however, the reproducibility was unsatisfactory. Methoxyphenamine was

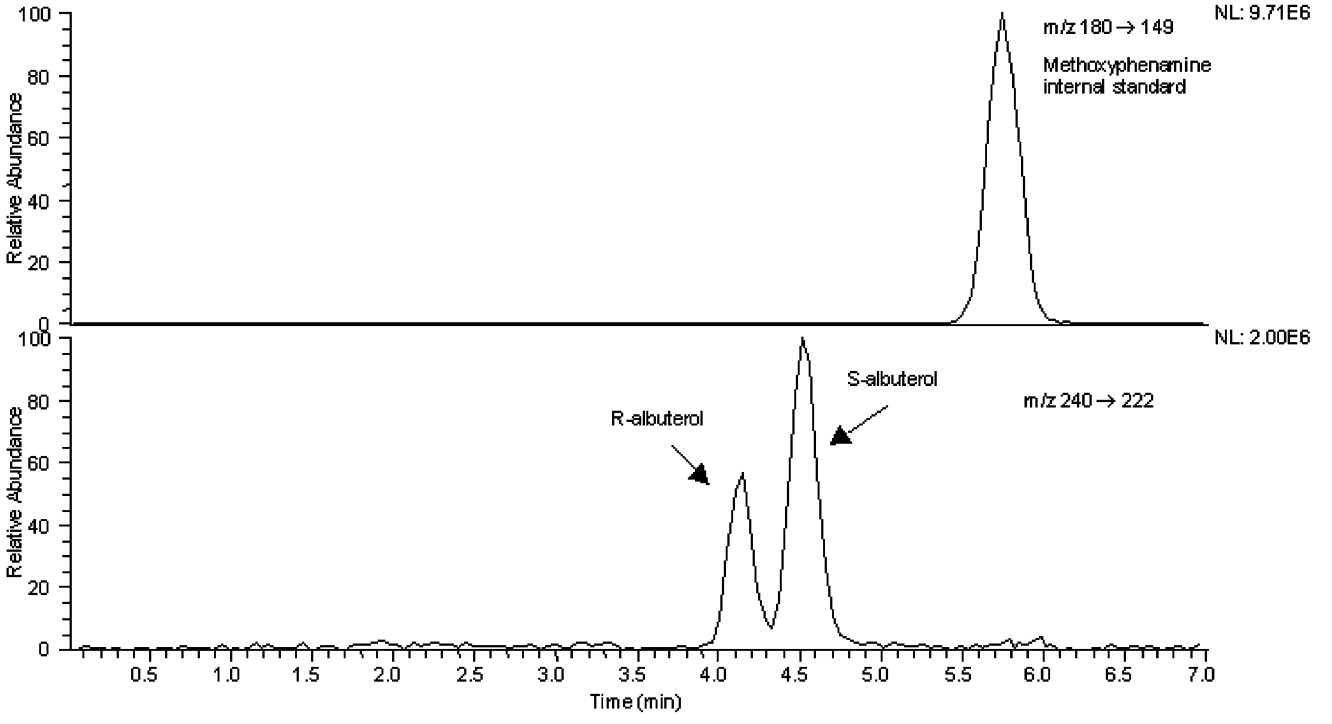

Fig. 2. Chromatogram of a plasma sample obtained from an asthma patient containing 3.2 ng/ml (*R*)-albuterol and 6.3 ng/ml (*S*)-albuterol.

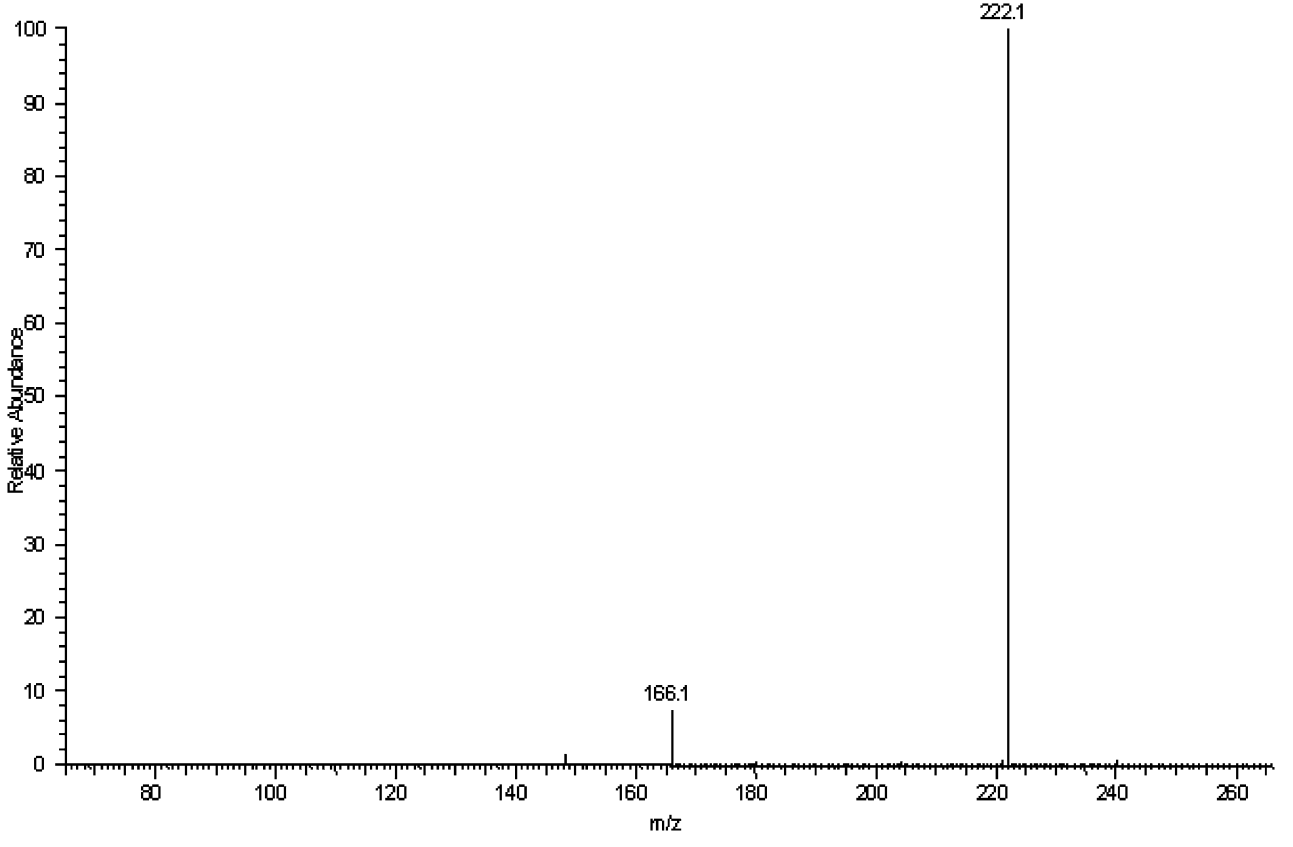

Fig. 3. Product ion mass spectrum from the albuterol $[M+H]^+$ ion at m/z 240 at a collision energy of 17% and 3 Da isolation window.

chosen as an internal standard; it is not available for therapeutic use in Australia, and so the risk of background plasma levels was negligible. Although methoxyphenamine possessed favorable chromatography characteristics such as peak shape and retention time, there was unsatisfactory variability in the peak area of methoxyphenamine due to retention on the SPE cartridge, a problem that would not be encountered if a deuterated albuterol standard was available.

An initial investigation of the retention of both albuterol and methoxyphenamine showed that approximately 30% of methoxyphenamine and 20% albuterol were still present on the cartridge after a 500 μl of methanol elution. After four consecutive 250 μl methanol elutions, greater than 95% of total albuterol and methoxyphenamine was recovered, although it was still found that the retention and subsequent use of methoxyphenamine added as an internal standard before the SPE procedure was unsatisfactory. By adding the methoxyphenamine after the SPE procedure and before blowing to dryness, the variability due to SPE was eliminated given that the albuterol recoveries appeared to be very consistent.

The reported sensitivity, recovery and reproducibility were sufficient to allow the assay to be used for pharmacokinetic determinations of very low levels of plasma albuterol enantiomers commonly seen after inhaled dosing. Inter-day comparisons were not examined as all analyses were carried out by batch analysis. Given the short run time of around 7 min, a batch of many samples including a calibration set and intra-day reproducibility samples could be carried out within a relatively short period.

5. Conclusion

This study demonstrates that the SPE and analytical techniques employed by Joyce et al. [12] are robust and can be modified and applied in a less specialized analytical laboratory environment by using a non-deuterated internal standard, manual SPE, and an ion trap LC–MS. These conditions still allow adequate assay sensitivity, recovery and reproducibility for the application to studies of inhaled albuterol.

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