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Rapid pharmacokinetic screening of salbutamol in plasma samples by column-switching high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

In order to obtain pharmacokinetic data from studies in humans, a sensitive and selective assay for the quantification of salbutamol in human plasma samples was required. This report describes an automated high-performance liquid chromatography–mass spectrometry assay with pre-column enrichment using internal standard calibration for the quantification of salbutamol and the validation of the assay. The lower limit of quantitation is 0.2 ng/ml with an accuracy and imprecision of less than 7%. The analysis time is 8 min per sample. © 1997 Elsevier Science B.V.

Keywords: Pharmaceutical chemistry; Column switching; Salbutamol; β-Agonists

1. Introduction

Salbutamol, a sythetic adrenergic amine, is a β-agonist and is therefore widely used for the treatment of pulmonary diseases in animals and humans. The ability of β-agonists to reduce protein catabolism and enhance lipolysis leads to an abuse of the drug. Farm animals were treated with β-agonist in the μg/kg range in order to enhance meat production. Such treatment resulted in residues in animal tissues [1–3] which can produce acute toxicity (cardiac, central nervous system, pulmonary) in humans consuming the liver of illegally treated cattle [4]. For less polar β-agonists several gas chromatographic (GC) methods in combination with electron impact ionization (EI) [5–8] and chemical ionization (CI) [9–12] were employed. Methods using thermospray in combination with supercritical fluid chromatography (SFC) [13] and liquid chromatography (LC) [14] are also described.

For the more polar compounds, HPLC–MS [15–20] methods were developed, but to our knowledge only a few contributions dealt with the measurement of β-agonists in complex matrices, while reaching the requirements for validation. In contrast to these papers we report a validated HPLC–MS method wherein sample analysis of human plasma down to a level of 0.2 ng/ml takes about 8 min, while its accuracy is between −2.8 and 5.0% and its imprecision is between 1.6 and 6.8%. This method combines the advantages of the column-switching technique [21] with atmospheric pressure ionization (API). The method requires only minimum efforts for the sam-

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ple preparation, while still retaining the potential to decrease the quantitation limit.

2. Experimental

Salbutamol was purchased by Sigma (No. S-8260, Lot 73F0007). The [$^2H_9$]salbutamol and the [$^3H_3$]salbutamol were synthesised by the Radiochemistry group, Boehringer Ingelheim. EDTA–plasma from six healthy humans (three male and three female) was pooled and used for the preparation of spiked calibration standards, quality control and blank samples.

2.1. Sample preparation

A volume of 25 μl of internal standard ([^{2}H]salbutamol, 300 ng/ml) was added to 500 μl human plasma. The resulting solution was mixed with 20 μl $HClO_4$ (conc.) and shaken vigorously on a cyclo mixer. The samples were then incubated for 10 min in an ice-water bath. The precipitated protein was separated centrifuging for 15 min at 12 000 *g* (Hettich Microliter centrifuge, Hettich, Tuttlingen, Germany). From the "protein free" supernatant, 240 μl were removed and mixed with 60 μl of a 10% ammonia solution. The pH of the resulting solution was between 8 and 9. A volume of 250 μl was injected. For testing linearity, concentrations of 0.2, 0.4, 0.6, 1,1.5, 3, 6, 10 and 15 ng/ml were employed. Concentrations of 0.5, 5 and 12 ng/ml were used in the quality control samples.

2.2. Recovery

In order to determine the yield after the protein precipitation a volume of 243.3 μl internal standard (I.S.) [^{2}H]salbutamol having a concentration of 300 ng/ml was added to 4865.5 μl plasma that contained [^{3}H]salbutamol (Lot No. Br 992/31) at a concentration of 30 ng/ml. A volume of 1.25 ml of the spiked plasma, equivalent to an activity of 33 688 dpm, was placed in an Eppendorf vial, where the protein was precipitated with 40 μl conc. $HClO_4$. The sample was immediately mixed on an cyclo mixer and incubated in an ice bath for 10 min. Then the sample was centrifuged for 10 min at about 12 000 *g*. The activity of the supernatant was measured. From the supernatant 500 μl were removed and mixed with 125 μl of a 10% NH_4OH solution for chromatography. From this solution volumes of 250 μl were used to determine the recovery after column-switching.

2.3. HPLC

After protein precipitation, salbutamol was extracted automatically by C_{18} reversed-phase extraction-columns. This extraction was performed according to the column-switching technique described by Roth and Beschke [21]. Fig. 1 displays a schematic flow diagram of the technical set-up of the HPLC equipment used for this assay. A volume of 250 μl of sample was injected into the sample loop of the auto sampler and washed onto the extraction column 8a (steel, 20 mm ×4.6 mm I.D., Bischoff, Leonberg, Germany, dry filled with Bondesil C_{18}, 37–50 μm, ICT, Frankfurt, Germany) with a flow-rate of 1.5 ml/min by pump A (Jasco Pu 980, Jasco, Tokyo, Japan). Upon flushing with buffer, the compound

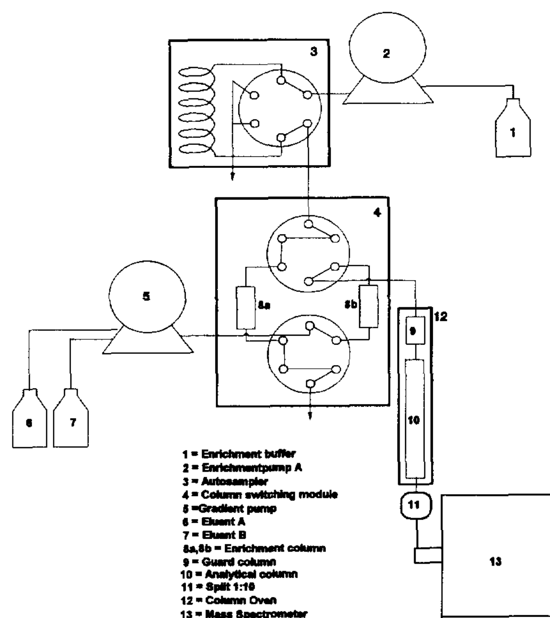

Fig. 1. Flow diagram of the HPLC–MS apparatus using the column switching technique for the automated extraction of salbutamol on the enrichment column.

was retained quantitatively on the extraction column (Fig. 1), whereas a large proportion of the plasma impurities, such as remaining proteins, were eluted by the enrichment buffer (10 m*M* ammonium formate solution, adjusted to pH 7.5 with ammonia solution 10%, flow-rate 1.5 ml/min). After 2.5 min of extraction, the two valves of the column switching module (Besta, Wilhelmsfeld, Germany) were switched. Substances retained on column 8a were eluted in the opposite direction from the extraction column and transferred onto the analytical column for separation (see Fig. 1). Then, the extraction column 8b was reconditioned with the enrichment buffer: the sample was separated isocratically with a flow-rate of 300 μl/min [0–5.5 min, acetonitrile–10 m*M* ammonium formate pH 5.0 (75:25), adjusted with formic acid] on the analytical column (125 mm×2.1 mm with guard column, 20 mm×2.1 mm, Bischoff, Leonberg, Germany, slurry packed with Purospher C_{18}, Merck, Darmstadt, Germany). The temperature of the column oven was 40°C. HPLC peaks of salbutamol eluting from the analytical column were detected after a split of 1:10 by a mass spectrometer, run in the MRM mode. The employed pump was a HP1090 ternary gradient pump with integrated autosampler and diode array detector, (Hewlett-Packard, Waldbronn, Germany).

2.4. Mass spectrometry

All data were recorded on a VG Quattro II, Fisons, Altrincham, UK, equipped with a API source and MRM chromatograms were acquired to disk. The source temperature was kept at 120°C, the nebuliser gas flow to 19 l/h and the drying gas flow to 300 l/h. The collision gas pressure was about $2.8 \cdot 10^{-3}$ mbar. The employed transitions were for salbutamol were 240 u to 148 u and 249 u to 149 u for the [$^2H_9$]salbutamol, which was used as internal standard (see Fig. 2).

2.5. Statistical parameters

Calibration was carried out by an internal standardisation method using linear regression. Calculations were performed using the Fisons masslynx 2.0 software package of the VG Quattro II Tandem mass spectrometer mentioned in Section 2.4.

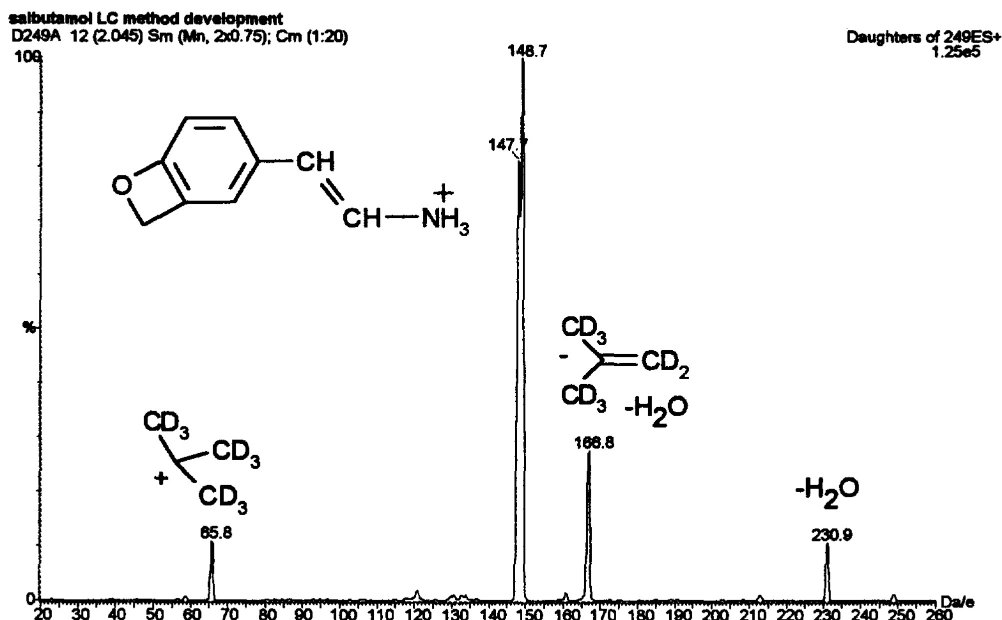

Fig. 2. Tandem mass spectrum of [^{2}H]salbutamol and a schematic fragmentation pathway of [^{2}H]salbutamol. Double peaks having a mass difference of only 1 u are due to proton scrambling during the fragmentation.

Salbutamol concentrations of 0.2, 0.4, 0.6, 0.8, 1, 1.5, 3, 6, 10 and 15 ng/ml were used for the calibration curve. Each concentration was determined five times. Regression analysis of the measured areas under the MRM signal peaks (AUP) on concentration was performed using the equation $y = a + bx$, where x = concentration and y = AUP. Weights used were $1/y$.

The inaccuracy was assessed by comparison of the measured and the theoretical values. Inaccuracy of the calibration curve was acceptable if relative deviations from the theoretical value were less than 15%.

The overall assay imprecision was calculated on the basis of the quality control samples obtained at different days for salbutamol concentrations of 0.5, 5 and 12 ng/ml.

3. Results

Fig. 3 shows a HPLC chromatogram of blank human plasma (pool of three male and three female) spiked with I.S.. In Fig. 4 the HPLC chromatograms of a plasma sample spiked with a low (0.2 ng/ml) concentration of salbutamol are depicted. The retention time of salbutamol was 4.9–5.1 min. Only neglectable interferences from background were observed.

Linear calibration curves were obtained upon injection of 250 μl of spiked plasma over a concentration range of 0.2–15 ng/ml salbutamol. The relative inaccuracy of the calibration samples were: 5% (0.2 ng/ml), 0.5% (0.4 ng/ml), −2.0% (0.6 ng/ml), −1.5% (0.8 ng/ml), 1.75% (1.0 ng/ml), −2.83% (1.5 ng/ml), 2.13% (3 ng/ml), −0.1% (6 ng/ml), 0.0% (10 ng/ml) and 0.07% (15 ng/ml) using weights $1/y$. The imprecision (R.S.D.) of the quality control samples calculated from ten-fold determinations of the was 6.74% (0.5 ng/ml), 1.64% (5 ng/ml) and 2.46% (12 ng/ml). Linearity and goodness of fit were evident. The regression equation of AUP values versus concentration was: $y = a + bx$ (y = AUP, x = concentration).

The resulting calibration curve using regression analysis of all five calibration curves, with weights $1/y$ had an intercept of 0.0105 and slope of 0.1821. The correlation coefficient was 0.9998.

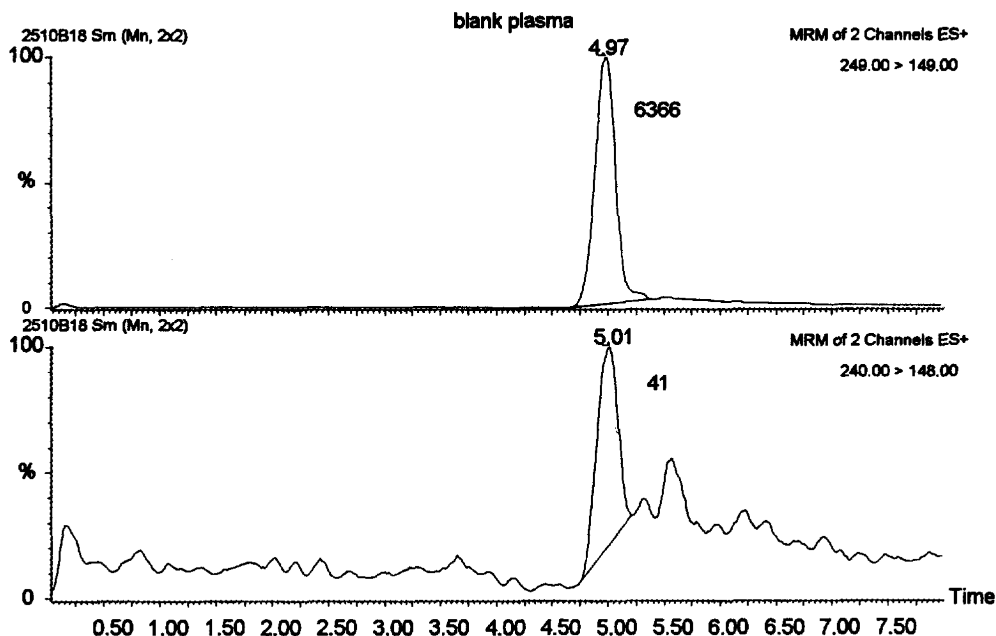

Fig. 3. HPLC chromatogram of blank human plasma spiked with I.S.. Ion trace [^{2}H]salbutamol (top) and of salbutamol (bottom, enlarged scale).

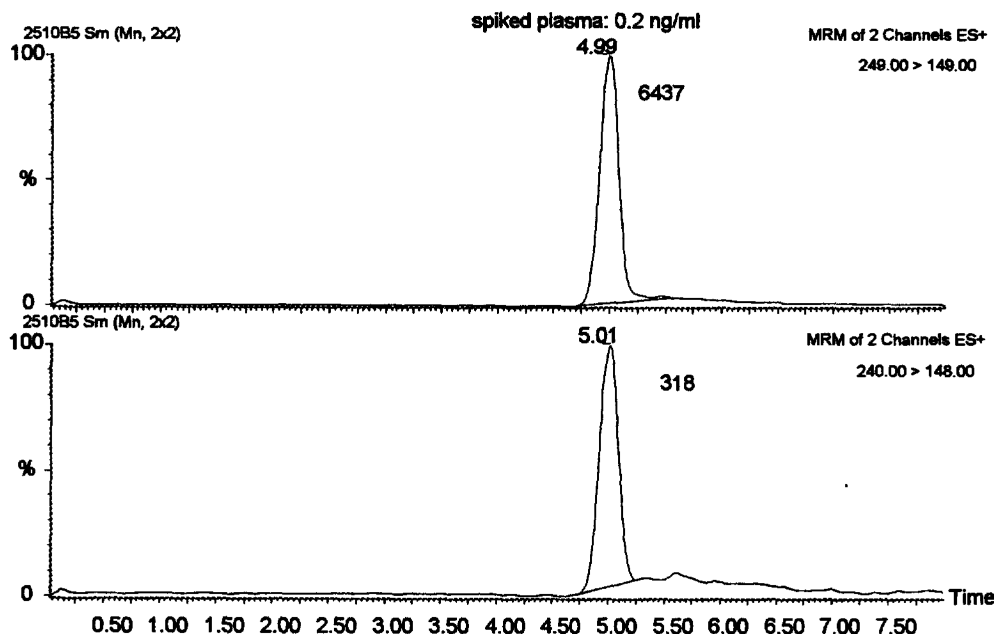

Fig. 4. HPLC chromatograms of human plasma spiked with 0.2 ng/ml salbutamol. Ion trace [^{2}H]salbutamol (top) and of salbutamol (bottom).

Using undiluted samples, the lowest standard concentration used in the calibration curve was 0.2 ng/ml and was therefore defined as the lower limit of quantitation. At this concentration the inaccuracy value was 5.0%. Using undiluted samples, the upper limit of the calibration curves was 15 ng/ml. At this concentration the inaccuracy value was 0.07%.

The recovery after protein precipitation was about 75.1% with a R.S.D. of 3.1%. The recovery after column switching was slightly above 100% indicating that the analyte is retained by the pre-columns.

The carry-over effect was investigated by injection of blank plasma after analysis of plasma samples with high concentration (10 and 15 ng/ml). The measured concentration values were below the limit of quantitation, which was 0.2 ng/ml. Therefore, a carry-over effect of <0.61% was calculated. Tiny signals observed in the background of blank plasma samples are most probably due to a small carry-over effect and minor impurities of the deuterated standard. However their peak areas are about factor 8 smaller than those of the smallest concentration. Thus, the assay is specific for salbutamol in the described concentration range. Furthermore, the MRM mode guarantees a very high specificity since only one single transition, characteristic for each substance, is able to give a signal in the chromatogram. Chromatograms of the highest and the lowest concentration as well as of a blank plasma spiked with internal standard are shown in Figs. 3 and 4.

4. Discussion

In this paper a fast, sensitive and reliable method for the quantitative determination of salbutamol in human plasma is presented. Nevertheless an even higher throughput and a better sensitivity can be reached. Due to the minimised sample preparation and the short run times, the screening of great amounts of samples for the presence of salbutamol can be performed in a rather short time. The high sensitivity of the mass spectrometer allows a limit of quantitation of less then 200 pg/ml sample which corresponds to an amount of 50 pg in total. One of the limiting factors to lower the limit of quantitation is the small sample volume injected on the pre-columns (250 μl). Raising the injection volume by

about 1 ml should give a 4-fold lower quantitation limit. By employing a shorter column as well as using the tandem switching technique for the pre-columns, the individual analysis time could also be shortened. The combination of HPLC–MS and column-switching proved to be a very efficient way for quantitative investigations. The set up allows a fast, exact and sensitive determination of drugs in biological matrix, with minimal effort for sample preparation needed.

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