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Rapid and automated determination of the β_2-agonist reproterol in human plasma by atmospheric pressure chemical ionisation high-performance liquid chromatography–tandem mass spectrometry

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Abstract

An on-line HPLC assay with tandem mass spectrometric detection for the fast and sensitive determination of reproterol in human plasma was developed, utilising a methylated structural analogue as the internal standard. Automated solid-phase extraction of diluted plasma samples, based on 250 μl plasma aliquots, at pH 6.5, allowed a reliable quantification of reproterol down to 400 pg/ml. Injection of 100 μl of plasma extracts onto a 30 mm×4.6 mm reversed-phase guard column provided retention times ranging from 20 to 30 s for reproterol and the internal standard. The standard curves were linear from 0.2 to 200 ng/ml using weighted linear regression analysis ($1/y^2$). The inter-assay and intra-assay accuracies were −0.9% and +3.2%, exhibiting a precision (C.V.) of ±11% and ±9.3%, respectively. Up to 100 unknowns may be analysed each 24 h per analyst. © 1997 Elsevier Science B.V.

Keywords: Reproterol

1. Introduction

Reproterol (marketed as Allergospasmin) is a potent β_2-agonist used for the treatment of allergic and non-allergic (intrinsic) asthma and chronic obstructive pulmonary disease (COPD) [1,2]. Studies on the pharmacokinetics, biotransformation and high-performance liquid chromatography (HPLC) with UV detection of reproterol were carried out by Niebch and coworkers in the late seventies [3,4].

The marketed formulation contains dichlorotetrafluoroethane ($C_2F_4Cl_2$) and dichlorodifluoromethane (CF_2Cl_2) in a metered dose aerosol inhaler (MDI) which itself caused problems for many patients trying to master this delivery technique [5]. In order to replace the ozone depleting chlorofluorocarbons (CFCs) alternative drug delivery systems with little or no effects on stratospheric ozone had to be invented and tested against the classical aerosol inhaler.

Hence, a new multiple dose powder inhaler (MDPI) has been developed, using micronized powder of reproterol (from a capsule) which is automatically carried along by an airstream during inhalation. For the purpose of comparing the pharmacokinetics of the MDI versus the MDPI formulation, a study including single and multiple dose administrations of both formulations was carried out in healthy volunteers.

Conventional quantitative HPLC with ultraviolet

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and fluorescence detection assays for reproterol showed insufficient sensitivity with limits of quantification above 50 ng/ml and 10 ng/ml of plasma, respectively [4]. Another reported analytical technique for the determination of reproterol in human plasma using HPLC in combination with amperometric detection on a rotating working electrode offered reasonable sensitivity of 1 ng/ml [6]. However, this method provided long chromatographic run-times and labour intensive workup procedures, which might not be favoured in large clinical pharmacokinetic studies.

On-line HPLC with tandem mass spectrometry (MS–MS) using atmospheric pressure chemical ionisation (APCI) in combination with automated solid-phase extraction (SPE) should provide the required sensitivity, straightforward sample workup and fast capability for trace level quantification of reproterol in human plasma.

Our aim was to develop a fast mass spectrometric method using on-line HPLC–APCI-MS–MS in order to be able to analyse reproterol in human plasma samples in the nanogram per millilitre range during an ongoing pharmacokinetic study in healthy volunteers. In order to enable us to report plasma concentrations to the clinical pharmacology unit as fast as possible we envisaged having an efficient sample workup procedure without time consuming sample concentration/redissolving and sample transfer steps.

2. Experimental

2.1. Materials

7-{3-[2-(2,5-Dihydroxyphenyl)-2-hydroxy-ethylamino]-propyl}-theophylline (I) (reproterol, Bronchospasmin, Allergospasmin) and 7-{3-[2-(2,5-dihydroxyphenyl)-2-hydroxy-ethylamino]-(1-methylpropyl)}-theophylline (II), the structural analogue, D-4908 (Fig. 1), used as internal standard, were synthesised within the Department of Chemical Research at ASTA Medica (Frankfurt, Germany). Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) and were of HPLC grade. All other reagents and chemicals were of either HPLC or analytical grade and were used without any further purification.

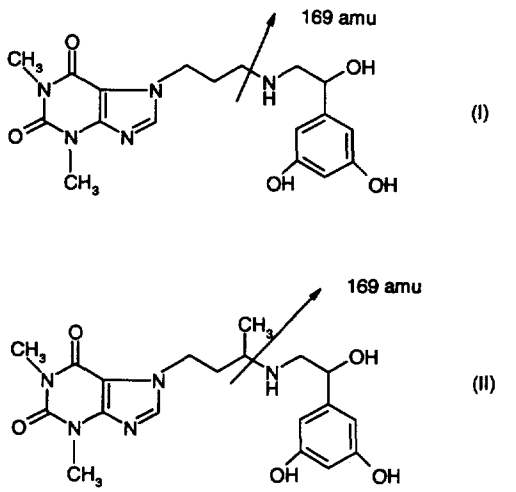

Fig. 1. Structures of reproterol (I) and the internal standard (II). The loss of 169 u yielding the major fragment ions is indicated.

2.2. Sample preparation

Blood samples were collected into heparinised or EDTA pre-treated tubes, cooled in ice and centrifuged for plasma (3000 *g*) as soon as possible (<1 h). Frozen samples were thawed at room temperature.

To 250 μl of plasma, 10 ng internal standard in 100 μl 0.01 *M* aqueous hydrochloric acid and 500 μl of ammonium acetate (0.1 *M*, pH 6.5) were added. In order to give a final volume of 1 ml, ammonium acetate (0.1 *M*, pH 6.5) was added to each vial, followed by brief vortex mixing. SPE was carried out by an automatic sample processor (ASPEC XL, Gilson, Villier-le-Bel, France) running overnight using 1 ml C_{18} Bond Elut SPE cartridges (Varian, Harbor City, CA, USA). Conditioning of the SPE cartridges with 1 ml each of acetonitrile and 0.1 *M* aqueous ammonium acetate (pH 6.5) was followed by loading of 1.0 ml of plasma dilution, washing with 0.4 ml of 0.0025 *M* aqueous ammonium acetate (pH 6.5)–acetonitrile (8:2, v/v) and elution with 0.4 ml of acetonitrile–0.0025 *M* aqueous ammonium acetate (pH 6.5) (8:2, v/v) (mobile phase). An automatic sample processor (ASPEC XL, Gilson)

was used to transfer the extracted samples to the appropriate HPLC autosampler vials.

2.3. HPLC–APCI-MS–MS

HPLC was performed on a Phenomenex Ultracarb 5 μm ODS (30) Guard column (30 mm×4.6 mm) (Torrance, CA, USA) using a Thermo Separation Products, ConstaMetric 4100MS series HPLC pump (Fremont, CA, USA) and a Waters 717plus autosampler (Milford, MA, USA). The injection volume was 100 μl onto the column. The mobile phase was aqueous–acetonitrile (80:20, v/v) containing 0.0025 *M* ammonium acetate (pH 6.5) and the flow-rate was 1.0 ml min^{-1}.

Mass spectrometric detection was carried out using a Finnigan TSQ 7000 triple-stage-quadrupole instrument (Finnigan MAT, San Jose, CA, USA) operating in the positive APCI mode. Selected reaction monitoring (SRM) was employed using xenon as collision gas at a pressure of approximately 1 mTorr (1 Torr=133.322 Pa), with a collision energy of 20 eV. The APCI-MS operating parameters can be summarised as follows: vaporiser temperature 475°C, sheath gas (nitrogen, 9.999% purity) at 50 p.s.i. (1 p.s.i.=6894.76 Pa), auxiliary gas (nitrogen, 9.999% purity) flow set at 10 units (exact flow-rate in l min^{-1} was not measured), discharge needle at 5 μA, heated capillary temperature at 180°C. Precursor to product ion transitions were monitored for *m/z* 390 to *m/z* 221 for reproterol (I), and for *m/z* 404.2 to *m/z* 235.2 for the internal standard (II) with a scan-time of 1.5 s.

Data were acquired by the Finnigan software (ISIS 8.1.1) and the peak areas measured using the Quan-software program. The mass spectrometer was shut down after overnight runs by instrument control language procedures (icl).

2.4. Preparation of calibration curve

Calibration samples and quality control (QC) samples were prepared with each batch of unknown test samples to cover the range of 0.2 to 50 ng/ml. To 250 μl of blank human plasma were added 0.05, 0.1, 0.25, 0.5, 1.25, 2.5, 5 and 12.5 ng of analyte (reproterol) in standard dilutions of 0.01 *M* hydrochloric acid in volumes of 25 μl. The internal standard (10 ng) was added to each tube in volumes of 100 μl. After brief vortex mixing, the samples were centrifuged and submitted to automated SPE as described in Section 2.2.

2.5. Preparation of quality control samples

QC samples were prepared by an independent analyst at two concentrations towards the top and bottom of the calibration curve (QC1: 1.5 ng/ml; QC2: 20 ng/ml). Two of each QC samples were stored for 6 months at −20°C and analysed together with the unknown test samples in every batch.

2.6. Quantification

Calibration curves were constructed by plotting peak area ratios of the analyte and the internal standard against the analyte's concentrations. The results of the raw data were transferred to the Finnigan quantification software (Quan: ISIS 8.1.1). Quan was used to calculate the weighted linear regression fit of the peak areas of the standards of reproterol relative to the internal standard. The weighted ($1/y^2$) linear regression line was fitted over the 250-fold concentration range. Drug concentrations in the unknown and quality control samples were calculated from this line.

3. Results and discussion

3.1. Mass spectrometry

Triple-quadrupole mass spectrometers with the APCI source for on-line HPLC–MS–MS are now routinely used for quantitative determinations of drugs and metabolites in the pharmaceutical industry. API interfaced to the pneumatically assisted electrospray (ionspray) and APCI are now routinely used for both qualitative (e.g., metabolite identification) and quantitative analyses. Electrospray is more likely to be utilised for the analyses of thermally labile compounds of biological origin, because of its extremely mild ionisation. However, the APCI mode appears to be favoured if thermal stability of analytes is assured, and high sample throughput assays with relatively crude sample work up procedures are

required [7,8]. Short retention times of analytes can also easily be established by using high flow-rates. APCI interfaces from various manufactures enable both optimum desolvation of excess solvent-(mixtures) by heated nebulisation, and ionisation through a corona discharge needle [9].

The lack of sufficient sensitivity of traditional HPLC–ultraviolet/fluorescence/amperometric detections in combination with labour intensive workup procedures was the driving force for the development of an HPLC–MS–MS assay with a simple, straightforward and automated SPE.

The deficiency of sufficient selectivity in single MS instruments made it necessary to use a triple-quadrupole mass spectrometric approach. We also envisaged by the application of tandem mass spectrometric detection to minimise the requirements for sample pre- treatment. In order to obtain an extracted plasma sample that is clean enough to enter the mass spectrometer's interface, SPE has already proved its suitability in combination with tandem mass spectrometric detection in many reported pharmaceutical and bioanalytical applications [10].

Fig. 2a shows the APCI product ion mass spectrum of reproterol (I) using the protonated molecular ion (m/z 390) as the precursor ion. The major fragment is formed by an α-cleavage (Fig. 1) with a loss of m/z 169 yielding the product ion used for quantification at m/z 221. The mass spectrometer's parameters (tune file) were set such to optimise the abundance of the major fragment ion (m/z 221), exhibiting the loss of the hydroxylated aromatic ethyl amine moiety.

The major product ion fragment of the internal standard (II) at m/z 235 used for the quantification is represented by the same loss of 169 u (Fig. 1). In Fig. 2b the APCI product ion mass spectrum of the internal standard (II) is displayed. Our approach in optimising the analytical product ions was to tune the mass spectrometer's parameters for relatively low mass resolution in the first mass analyser Q1 (Δm_{Q1} of the protonated molecular ion at half peak height was 2 u), and for higher mass resolution in the second mass analyser Q3 (Δm_{Q3} of the product ion at half peak height was 0.6 u). Hence, a gain in sensitivity was achieved by setting the resolution of the first mass analyser to approximately 2 Daltons. This approach in tuning the mass spectrometer has proved to be suitable for the quantification of reproterol since negligible mass "carry-over" from the plasma matrix (proteins and salts) or the internal standard was observed, operating in the SRM mode. In Fig. 4 a typical product ion chromatogram for a blank plasma sample ("spiked" with internal standard) is depicted, illustrating the mass contribution(s) for both, the matrix and the internal standard of approximately 1% for the lowest standard.

3.2. Chromatography

In order to develop an assay with high sample throughput and to obtain maximum response for the analytes we required as short a retention time as possible. The great advantage of having analytes with different molecular masses and similar retention times in combination with tandem mass spectrometric detection, enabled us to aim at a minimum separation that might remove some salts or matrix components that can suppress or interfere with the analyses from the target components, while maintaining good sample throughput. The "chromatographic aim" was to localise the analyte and the internal standard at a short retention-time without the need to implement a real chromatography that would have lead to longer retention- and run-times. Previous experiments have shown that it is paramount to implement at least such a crude minimal chromatographic separation of reproterol for adequate sensitivity and to prevent blockages of the heated capillary system and the front end of the mass spectrometer. Another important reason why the sample should be separated from some contaminants is that the reactant ions may be depleted when sample and large excess of other components coelute without any guard column. As a consequence proper formation of protonated sample and internal standard molecules could no longer take place, and quantification with reproducible precision and accuracy might not be feasible [11].

Furthermore, APCI-HPLC–MS interfaces from various manufactures differ in the way they prevent contamination of the high vacuum region of the mass spectrometers' from non-ionisable particles and solvents. Hence, enabling the transfer of ions in the gas phase from atmospheric pressure into the high vacuum region towards the detector (electron multi-

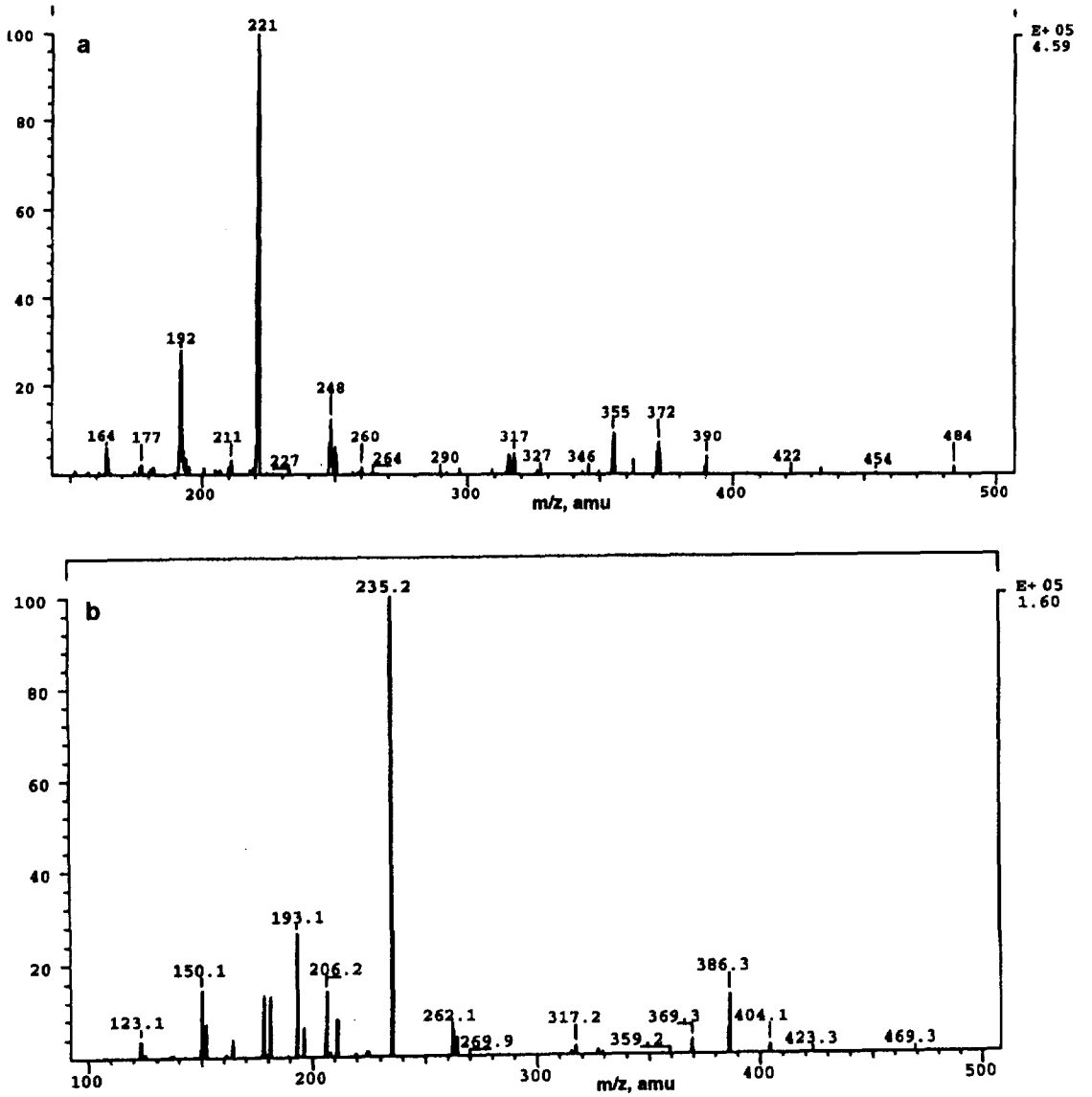

Fig. 2. (a) Positive product ion mass spectrum of the reproterol precursor ion at m/z 390; ~40 pg presented to the source (APCI-HPLC–MS–MS mode). (b) Positive product ion mass spectrum of the internal standard (II) precursor ion at m/z 404; ~40 pg presented to the source (APCI-HPLC–MS–MS mode).

plier). When using a heated capillary device interfacing the atmospheric pressure region with the high vacuum sector, especially with a rigid sprayer onto the capillary's aperture at nebuliser temperatures above 400°C, blocking of the front end of the heated capillary appears to be likely. Subsequently, a significant loss in sensitivity occurred on our mass spectrometer in combination with our sample pretreatment, due to accumulations of non-volatile residues upcoming from the biological plasma matrix. In that regard, we observed a major drawback of our reported assay associated at high flow-rates when running in the APCI mode. It must be mentioned that we primarily designed our assay to enable us a high

sample throughput and short chromatographic runtimes. Therefore, we allowed the sample pre-treatment to produce relatively crude plasma extracts and having only a 3 cm guard column for a "minimum chromatography". The problem of premature blocking of the heated capillary could be partially mastered by using a post-column stainless-steel filter (pore size: 0.5 μm). Nevertheless, frequent mechanical cleaning of the heated capillary device was essential, even during analytical runs, to maintain acceptable assay performance at least for the standard curve above 0.4 ng/ml. Longer sized analytical columns (10 to 20 cm) might have also prevented early blocking of the heated capillary, but it appeared to us to be the better compromise using a short column technique in order to implement high sample throughput. The drawback of premature blocking of the heated capillary interface could have been less dramatic by using interfacing techniques that operate with "off-axis" or orthogonal spraying of the eluates onto the aperture of the mass spectrometer which itself is shielded from atmospheric pressure by a nitrogen curtain gas interface [12]. Unfortunately, such a device was not available for us at that time. After running the reproterol plasma samples that had undergone the same SPE cleanup procedure and the same guard-column, chromatographic setup, but on a triple quadrupole mass spectrometer with an "off-axis"–spraying interface, we have now proof that the front-end of this mass spectrometer after continuous use for 6 days (at least 12 h per day) is much less likely to get blockages at its aperture. Therefore, relatively crude plasma extracts may be analysed on such an instrument (publication on this topic in preparation).

A further important factor of having high sample throughput analyses for reproterol is based on its lack of stability as a solid and in plasma samples (stability guaranteed for approx. 10 to 12 h) [4]. We observed a stability of diluted plasma samples "spiked" with reproterol of approximately 10 h at room temperature. Therefore, the stability requirements for automated and unattended SPE of diluted reproterol plasma samples were satisfactory. Automated, unattended SPE took for 100 plasma samples approximately 10 h.

After testing several of the immense variety of short columns available on the market, we obtained the best results regarding short retention times with the Phenomenex Ultracarb 5 μm ODS (30) 30 mm× 4.6 mm I.D. guard column. This column provided us with the best compromise in terms of chromatographic reproducibility, flow-rate, high sample throughput and, last but not least, cost effectiveness. One column was used for approximately 200 samples without deterioration, such as peak tailing and decreasing signals, which we received after the injection of approximately 300 extracted plasma samples. In order to prolong the life-time of the guard columns, efforts were made by washing these with acetonitrile, acetonitrile–water (1:1, v/v), methanol and methanol–water (1:1, v/v). As a result, the columns could not be used for further analyses even after washing with acetonitrile by reversed column flow. This might also indicate that the plasma sample extracts analysed after SPE are fairly crude and cause a relatively fast deterioration of the stationary phase.

The lack of a suitable stable labelled form of reproterol, forced us to implement another, structurally related derivative. The reasons for choosing (II) as the internal standard were its chromatographic and mass spectrometric similarity to reproterol in terms of the lipophilicity and recovery from biological matrices after SPE from C_{18} SPE cartridges [recovery of reproterol and (II) ≥90%]. The internal standard (II) carries only one additional methyl group at the α-position of the secondary amine function (Fig. 1). The fact that (II) has already been used successfully as an internal standard in a previously published reproterol HPLC assay reassured its chromatographic suitability [4].

Selected ion chromatograms of a plasma extract from a blood sample taken 2 h after dosing (MDI) of 1 mg reproterol to a healthy volunteer, are shown in Fig. 3. Selected ion chromatograms of a blank plasma sample "spiked" only with (II) are given in Fig. 4. Typical chromatograms of a standard at the limit of quantification (LOQ: 400 pg/ml) are presented, revealing already visible baseline noise for the analyte (I) (see top trace in Fig. 5). Nevertheless, we kept this standard for performance calculations since we regarded its assay performance as still acceptable (see Tables 1 and 2).

The analyte and the internal standard nearly coelute with a retention time of approximately 30 s.

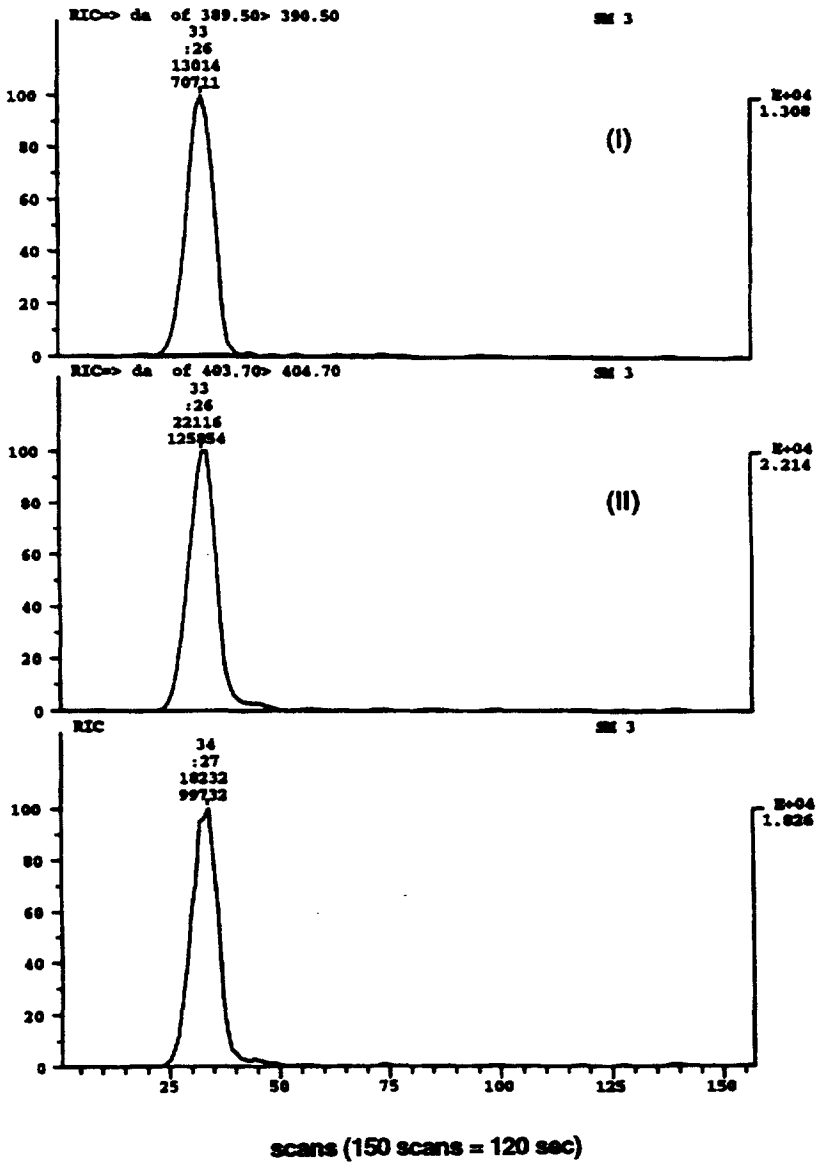

Fig. 3. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of reproterol (I) and the internal standard (II) in plasma extracts of a blood sample taken from a healthy volunteer 2 h after oral administration of 1 mg reproterol (MDI). The signals were generated by collision-induced dissociation of the protonated forms of reproterol (I) and the internal standard (II) at m/z 390 and 404, respectively. The derived concentration of reproterol was estimated at ~6 ng/ml plasma.

The cycle time per sample was 2 min. The detector response for reproterol was linear over the range from 0.2–50 ng/ml and even up to 200 ng/ml (the 200 ng/ml values are not displayed, but were archived with the raw data). It must be also emphasised that only in three out of fourteen analytical series the 0.2 ng/ml standard achieved an acceptable assay performance (relative errors ≤20%) and could therefore not be included for assay performance calculations (Table 1).

Typical calibration curves for reproterol comprised a mean slope of 0.0789 [n=4; relative standard

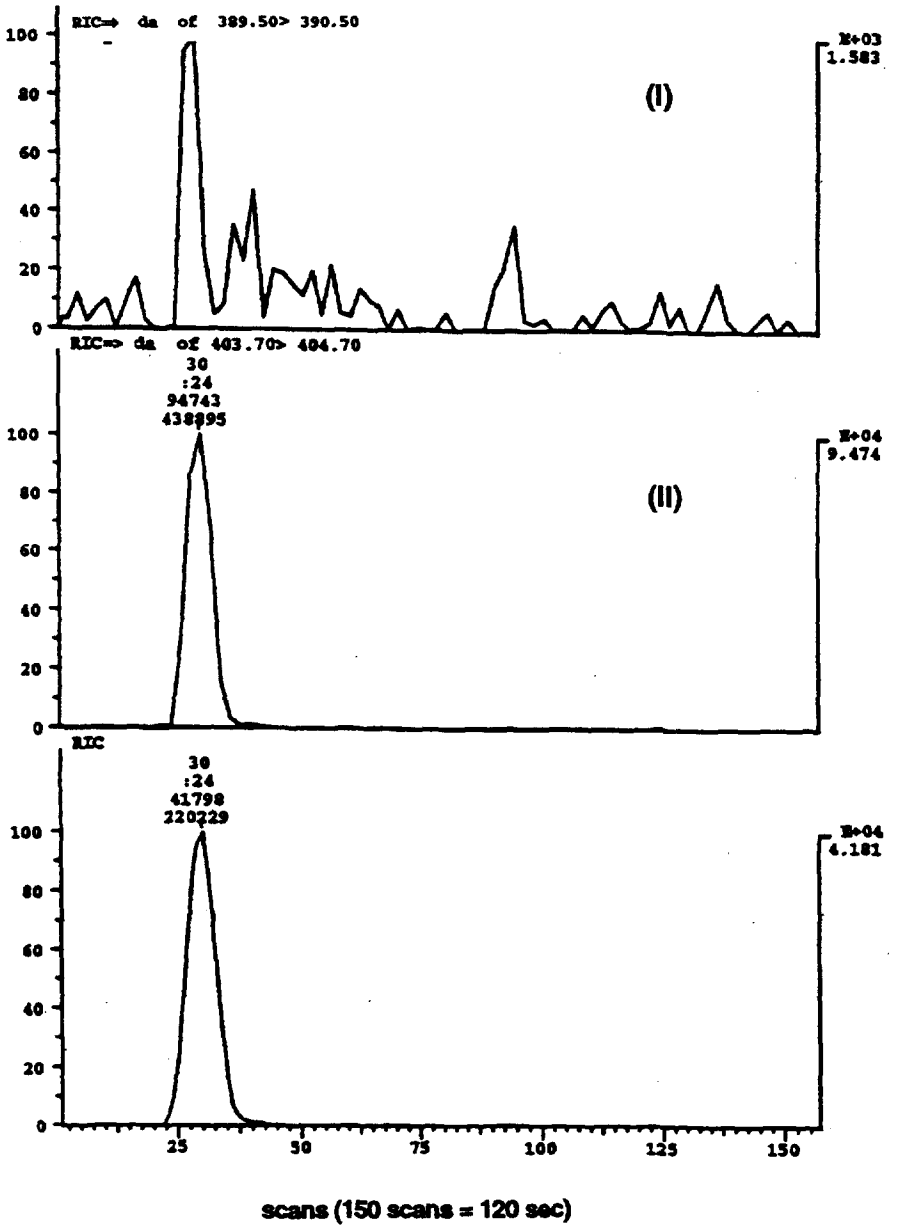

Fig. 4. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of reproterol (I) and the internal standard (II) in plasma extracts of a blank plasma sample "spiked" with (II) at a concentration of 40 ng/ml. The signals were generated by collision-induced dissociation of the protonated forms of reproterol (I) and the internal standard (II) at m/z 390 and 404, respectively.

deviation (R.S.D.)=0.001] with a mean intercept of 0.00465 (R.S.D.=0.0003) and a correlation coefficient (r^2) of 0.995. In order to evaluate the inter-assay performance of the analytical method we prepared and analysed standard curves on four subsequent days with four QC samples with each standard curve (duplicate QC samples towards the lower and duplicate QC samples towards the upper quartile of the calibration range). The intra-assay performance was assessed to ensure that the results

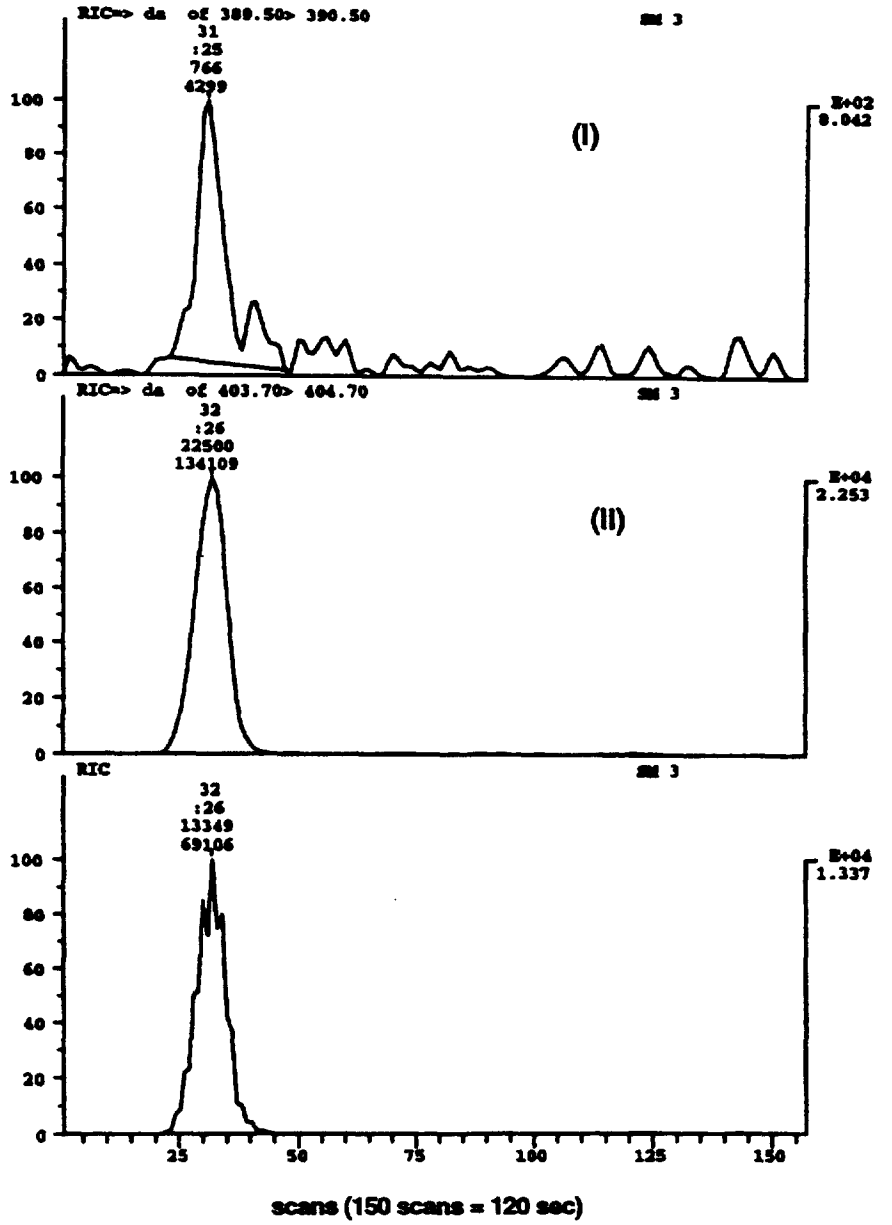

Fig. 5. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of reproterol (I) and the internal standard (II) in plasma extracts from a standard of the calibration curve at the limit of quantification (LOQ: 400 pg/ml) and the internal standard at a concentration of 40 ng/ml. The signals were generated by collision-induced dissociation of the protonated forms of reproterol (I) and the internal standard (II) at m/z 390 and 404, respectively.

were acceptable, and could be used for pharmacokinetic analysis. The mean inter-assay precision (C.V.) for the standards of reproterol in plasma was ±10.9% with a mean error of −0.9%, and for the QC samples ±8.8% with a mean error of +1.25% (Table 1). The mean intra-assay precision (C.V.) for the standards of reproterol in plasma was ±9.3% with a mean error of +3.2%, and for the QC samples

Table 1
Inter-assay performance for the determination of reproterol[a]

True value (ng/ml)	n	Calculated concentration (ng/ml)	Error (%)	C.V. (%)
0.20	3	0.17	−16.00	19.93
0.40	8	0.40	+1.05	9.84
1.00	12	1.05	+5.46	15.43
2.00	12	1.96	−1.99	7.07
5.00	12	5.10	+2.00	9.85
10.00	13	10.19	+1.86	10.06
20.00	13	19.96	−0.20	8.24
50.00	14	50.35	+0.71	7.30
			−0.89[b]	±10.97[c]

[a] Inter-assay (between-day) performance of reproterol standards.
[b] Mean error.
[c] Mean C.V.
Inter-assay (between-day) performance of QC's: QC1 (1.5 ng/ml) (n=45): 1.605 ng/ml±0.13; mean error (%): +7.0; C.V. (%): ±7.9. QC2 (20 ng/ml) (n=42): 19.108 ng/ml±1.86; mean error (%): −4.5; C.V. (%): ±9.7. Mean error and mean precision of all calculated QC's: +1.25% and ±8.8%, respectively.

±13.7% with a mean error of −2.45% (Table 2). Therefore, the overall inter- and intra-assay performances for the standards and QC samples were satisfactory for routine on-line HPLC–MS analysis. Only the bottom standards at 0.2 ng/ml and occasionally at 0.4 ng/ml could not meet the acceptance criteria with an intra- and inter-assay precision (C.V.) ≥±20%, respectively. Subsequently, the standard at 0.2 ng/ml was excluded for further routine analyses of reproterol.

4. Conclusions

In conclusion, a very fast, sensitive, selective and robust assay with tandem mass spectrometric detection has been developed. The results of the assay performances clearly show that an assay for reproterol in plasma with a limit of quantification at 400 pg/ml could easily be established utilising a relatively rough automated sample cleanup procedure by SPE. This method has been employed successful-

Table 2
Intra-assay performance for the determination of reproterol[a]

True value (ng/ml)	n	Calculated concentration (ng/ml)	Error (%)	C.V. (%)
0.20	4	0.23	+15.26	23.93
0.40	4	0.39	−3.64	19.64
1.00	4	1.16	+15.83	6.37
2.00	4	2.01	+0.53	6.21
5.00	4	5.10	+1.95	5.86
10.00	4	10.14	+1.43	4.89
20.00	4	20.03	+0.17	1.95
50.00	4	47.10	−5.79	5.74
			+3.2[b]	±9.3[c]

[a] Intra-assay (within-day) performance of reproterol standards.
[b] Mean error.
[c] Mean C.V.
Intra-assay (within-day) performance of QC's: QC1 (1.5 ng/ml) (n=8): 1.70 ng/ml±0.17; mean error (%): +13.3; C.V. (%): ±10. QC2 (20 ng/ml) (n=8): 16.35 ng/ml±2.84; mean error (%): −18.2; C.V. (%): ±17.4. Mean error and mean precision of all calculated QC's: −2.45% and ±13.7%, respectively.

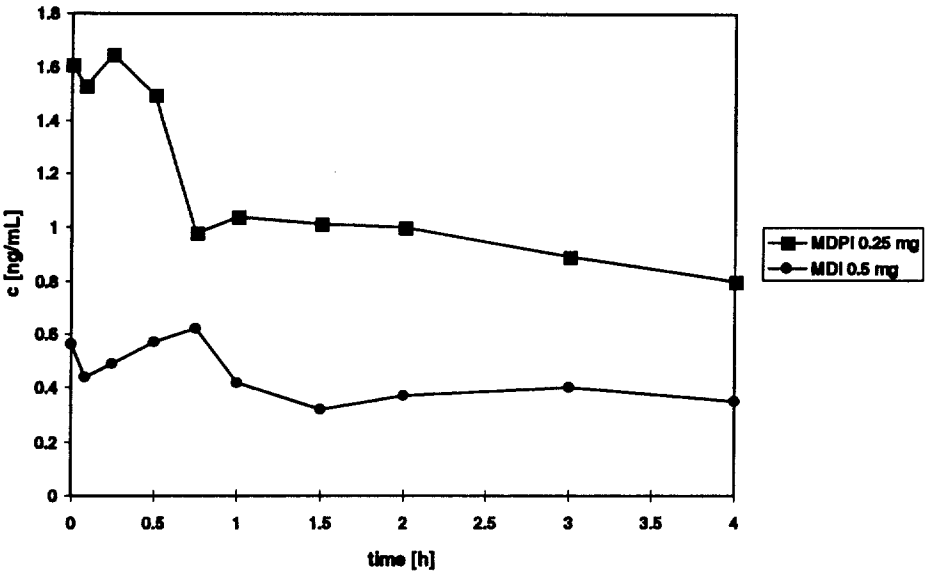

Fig. 6. Plasma concentration–time profile after oral administration (inhalation) of a single dose of 0.25 mg reproterol by MDPI (powder inhaler) and 0.5 mg MDI (aerosol inhaler) to a healthy volunteer.

ly for the determination of the pharmacokinetics of the β_2-agonist reproterol in a clinical pharmacokinetic study, in which volunteers received single and multiple doses of 1, 0.5 and 0.25 mg via MDPI and 0.5 mg via MDI administration. An example of a concentration versus time profile, in which drug concentrations were measured up to 4 h for reproterol after inhalation is outlined in Fig. 6. It could be demonstrated that elevated plasma levels were achieved with the new MDPI formulation versus the classical aerosol formulation.

Once again, HPLC–MS–MS with an APCI-interface has demonstrated itself to be an excellent tool for fast analysis of non-volatile, low-molecular-mass compounds in pharmacokinetic studies.

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