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# Ultra-sensitive determination of Formoterol in human serum by high performance liquid chromatography and electrospray tandem mass spectrometry

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**Abstract**

An analytical method was developed and validated to determine Formoterol in human serum in the range from 0.40 to 100.24 pg/mL by high performance liquid chromatography and tandem mass spectrometry (HPLC–MS/MS) due to the lack of efficient methods to determine very low levels of Formoterol in serum and plasma. Serum was diluted by water and mixed with the internal standard (d6-Formoterol). Formoterol and internal standard were extracted using a cation-exchange solid phase column (SCX-3). After eliminating endogenous serum constituents through washing steps with water and methanol, elution took place using methanol/ammonia. After evaporation of the elution liquid the residue was redissolved and analyzed by HPLC–MS/MS with electrospray ionisation (ESI) in positive mode. A gradient between 10 mM ammonium formate and acetonitrile was used. The inter-batch precision of the calibration standards ranged from 1.55% to 9.01%. The inter-batch accuracy of the calibration standards ranged from 93.37% to 107.30%. The lower limit of quantitation (LLOQ, 0.40 pg/mL) had a precision of 19.67% and an accuracy of 96.78%. Comparable results were obtained for quality control samples. Stability in human serum was given over three freeze/thaw cycles and 2 h at room temperature. Formoterol in human serum was stable for at least 6 months below −20 °C. This method has been used widely for quantifying Formoterol after inhalation of 9–36 μg of the drug by volunteers. A cross validation with human plasma versus serum was performed after this method was successfully validated in human serum.


*Keywords:* Formoterol; Human serum; Ultra sensitive method; Tandem mass spectrometry; Solid phase extraction; Liquid chromatography

## 1. Introduction

Formoterol is a potent selective ß2-adrenoceptor agonist. The determination of Formoterol in human serum and plasma required a very sensitive method due to the fact that only very low concentrations were available for analysis. After the application of 120 μg (inhaled) Formoterol, the maximum concentration value ($C_{max}$) was about 80 pg/mL [1]. For some studies much lower dosages down to 9 μg should be applied and the pharmacokinetic profile should be monitored. Therefore, a LLOQ of < 1 pg/mL was required.

Up to now, only a few published methods exist regarding the determination of Formoterol in human serum or plasma: a HPLC method with an electrochemical detector [2] with a LLOQ of 20 pg/mL by using 2 mL of plasma and another method with the same equipment [1] with a LLOQ of 4 pg/mL but under time consuming conditions (4 days HPLC before starting, 25 min per run). Some former published methods with GC–MS had higher LLOQs [3,4]. Another method used HPLC–MS/MS with ESI to determine Formoterol in urine to a LLOQ of 25 ng/mL [5]. Another approach used GC–MS [6] after derivatization and achieved a LLOQ of 0.5 ng/mL in urine.

We screened published methods for other β-agonists with a similar structure and obtained the following results: Procaterol was only determined in urine [7] (LLOQ at 4.3 ng/mL), Salmeterol was only determined in urine [8] with HPLC–MS/MS, for Bitolterol and Pirbuterol no methods were published for

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neither plasma nor urine, Fenoterol was determined in plasma after derivatisation with HPLC-fluorescence [9] to a LLOQ of 376 pg/mL or HPLC–MS/MS to a LLOQ of 2.5 ng/mL, Salbutamol (Albuterol) was determined with HPLC–MS [10] with a LLOQ of 100 pg/mL or GC–MS [11] with a LLOQ of 2 ng/mL or HPLC-fluorescence [12,13] with a LLOQ of 50 pg/mL, Terbutaline was determined with HPLC–MS/MS [14] with a LLOQ of 1 ng/mL or HPLC electrochemical detection [15] with a LLOQ of 0.8 ng/mL. All published methods regarding Formoterol related substances have a LLOQ between 50 pg/mL and 5 ng/mL in plasma or urine except Formoterol itself for which a LLOQ from 4 to 20 pg/mL was achieved with very time consuming methods [1,2]. The approach used by the authors is a combination of specific sample preparation on an ion exchange pre column (SCX 3) and a very sensitive HPLC–MS/MS system (API 4000). A sensitive method was established to a LLOQ of 0.40 pg/mL using a human serum or plasma volume of 1 mL.

## 2. Experimental

### 2.1. Chemicals

Formoterol was provided as Diformoterolfumarate ((±)-2′-hydroxy-5′-[(*RS*)-1-hydroxy-2-[[(*RS*)-*p*-methoxy-α-methylphenethyl]amino]ethyl]formanilide × 1/2 fumaric acid) by ALTANA Pharma AG (Germany). d6-Formoterol (deuterium labels on the methoxyphenyl group), used as internal standard, was supplied by the Technical University of Vienna (Prof. U. Jordis, TU Vienna, Austria). Methanol (purity: pro analysi) was obtained from Merck (Germany). Ammonium formate (purity >97%) and ammonium hydroxide solution (purity: puriss., about 25% in water) were obtained from Fluka (Switzerland). Dimethylsulfoxide (HPLC grade) was obtained from Riedel de Haen (Germany). The SPE-cartridges (SCX-3, 100 mg, volume 1 mL) were obtained from Separtis (Germany). Purified water (ASTM-I grade) was produced in-house. Human serum and plasma were pooled by pharm-analyt deriving from different volunteers. See Fig. 1.

### 2.2. Sample preparation

Serum samples were stored between −20 and −30 °C. After thawing in a water bath (about 20–25 °C) samples were portioned into aliquots of 1 mL.

These aliquots of serum were diluted with 1 mL of purified water and 50 μL of the internal standard working solution (∼1.5 ng/mL of d6-Formoterol in 20% methanol) and were transferred onto the preconditioned SCX-3 cartridges (preconditioning with 1 mL of methanol and 1 mL of water). The cartridges were washed with 1 mL of water and 1 mL of methanol. The analyte was then eluted using 1 mL of methanol/25% ammonia (95:5, v/v) into conical centrifuge vials. All precondition, sample transfer, washing and elution steps were performed by centrifuging the SCX-3 cartridge at about 140 g for 2 min (Centrifuge Megafuge, Heraeus, Germany).

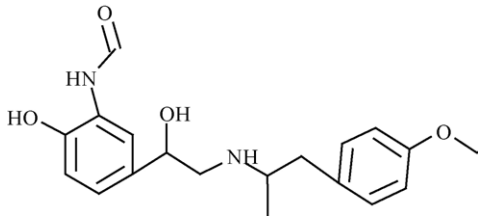


Fig. 1. Structure of Formoterol.

The eluted samples were evaporated for 10 min in a TurboVap at about 50 °C (Zymark, Switzerland). The residue was redissolved in 50 μL of 20% methanol/80% 0.01 M ammonium formate and vortexed four times for about 5 s and transferred into conical auto sampler vials for analysis.

### 2.3. High performance liquid chromatography

The HPLC system consisted of two PE Series 200 Micro Pumps (Perkin-Elmer, USA), a PE Series 200 Auto sampler (Perkin-Elmer, USA) and a Jetstream 2 Plus (W.O. Electronics, Austria) column oven. A Synergy Polar RP (100 mm × 2 mm, 4 μm) column (Phenomenex, USA) was used for separation.

The mobile phase consisted of Solvent A (10 mM ammonium formate in water) and Solvent B (50% 20 mM ammonium formate in water/50% acetonitrile). The flow rate was set to 0.8 mL/min. Solvent B was increased from 20% to 80% in the time range from 0.0 to 3.0 min (linear gradient). At 3.0–3.6 min isocratic conditions were run at 80% B. Re-equilibration was performed from 3.6 to 4.7 min at 20% B. The column temperature was 40 °C. The injection volume was 20 μL. The auto sampler was flushed five times with 250 μL of 67% acetonitrile.

### 2.4. Apparatus

A Sciex API 4000 (Applied Biosystems, Canada) triple quadrupole mass spectrometer was used for detection. Ionisation was performed using the ESI source in the positive multiple reaction monitoring (MRM) mode. The vaporiser temperature was set to 650 °C. The ionisation voltage was set to 4500 V. Nitrogen was used for curtain gas (setting 40 psi), nebulizer gas (setting 60 psi) and heater gas (setting 70 psi). The MRM transitions were 345.3 → 149.1 *m/z* for Formoterol and 351.3 → 155.1 *m/z* for d6-Formoterol. The collision energy was 27 V. Quadrupole resolution was set to unit → unit. The CAD gas setting was 5 psi and the declustering potential was 40 V. The HPLC and MS/MS were controlled using the PE Sciex Analyst 1.2 software. See Fig. 2.

### 2.5. Method validation

The analytical method was validated in three batches (including demonstration of linearity, accuracy, precision, specificity, recovery and LLOQ). A minimum of one set of calibration standards and five sets of quality control samples were analysed within these three different batches as well as a carryover, a blank, and a zero sample. Calibration standards were made at eight concentration levels by adding defined volumes of aqueous solutions containing Formoterol or a higher concentrated

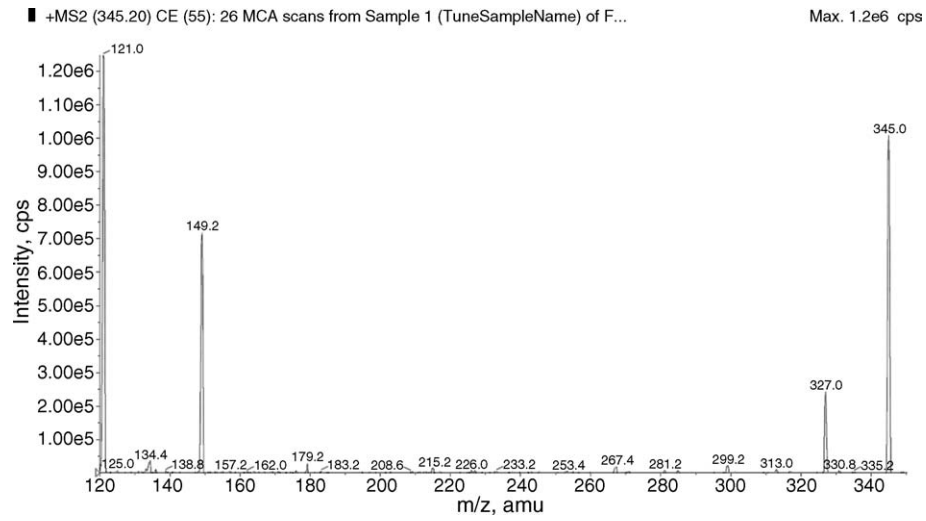


Fig. 2. Product ion scan for 345.2 *m*/*z* (collision energy 55 V), non-optimised spectrum, daughter ion taken for quantitation: 149.2 *m*/*z*.

calibration standard to analyte-free human serum. Quality control samples were prepared alike but spiked in a different batch of serum with different solutions of Formoterol deriving from a second weighing. Concentrations of calibration standard were between 0.40 and 100.24 pg/mL of Formoterol in human serum and at 1.10/6.00/84.86 pg/mL for quality control samples (serum and plasma).

To determine the assay's linearity, precisions (coefficient of variation, CVs) and accuracies at least three batches should be analysed, each consisting of at least one set of calibration standards, a zero sample (or Standard 0), a blank sample and five QC-samples at each of three concentration levels.

Intra- (for QC-samples only) and inter-batch (for calibration standards and QC-samples) precisions (as CVs in [%]) and accuracies (in [%]) of the assay should be derived from the results of the validation batches mentioned above.

Concentration values of both the calibration standards and the QC-samples should be back-calculated from the appropriate calibration curve. Thereof inter-batch mean values, precisions (as CVs) and accuracies should be calculated.

### 2.6. Specificity

Acceptable specificity was defined as an area of possible interferences in serum or plasma in blank and zero samples. "Blank sample" refers to Standard 0 without analyte and internal standard and "zero sample" refers to Standard 0 with internal standard. Blank and zero samples had to be below 1/3 of the area of Calibration Standard 1 (at level of LLOQ) or not detectable. The specificity of the method was determined by analysing six sample pairs consisting of one zero and one blank human serum sample per volunteer.

### 2.7. Recovery

Recovery was determined by comparing the areas of quality control samples with areas of aqueous solutions (without sample preparation but appropriate dilution in accordance to sample preparation of QC-samples) at three concentration levels. Each peak area of the QC-samples was divided by the mean peak area of those direct aqueous solutions. Aqueous DIR-samples had to be analysed in triplicate at each of these concentration levels.

### 2.8. Method linearity

The calibration range was from 0.40 to 100.24 pg/mL of Formoterol in human serum. The inter batch coefficient of variation had to be <15% (20% at LLOQ level) for precision, and for accuracy the mean value had to be within ±15% of the actual value (20% at LLOQ level). However, at LLOQ level, 20% was acceptable for both inter batch precision and accuracy. If the calibration curve was rejected, the batch had to be rejected also. A linear regression should be used with a weighting factor of $1/x$. The coefficient of correlation ($R$) has to achieve a degree of certainty of $R = 0.99$.

Accuracy of individual calculated values must not deviate more than ±15% (20% at LLOQ level) from their expected ones.

Seventy-five percent of all individual values, but at least six concentration levels have to match the specifications mentioned above.

If any values failed (⟨oos⟩), the respective calibration curve will be calculated anew without them, retaining the upper conditions unchanged. At least 50% of all individual values at a certain concentration level have to be valid, else this concentration level fails (⟨oos⟩).

⟨oos⟩ concentration levels are allowed unless those being adjacent ones.

### 2.9. Precision and accuracy for QC samples

Five replicates of quality control samples at three concentration levels each had to be analysed. Quality control samples were prepared at three concentrations (≤3 × LLOQ, mid-range and at least 80% of the highest calibration concentration) and

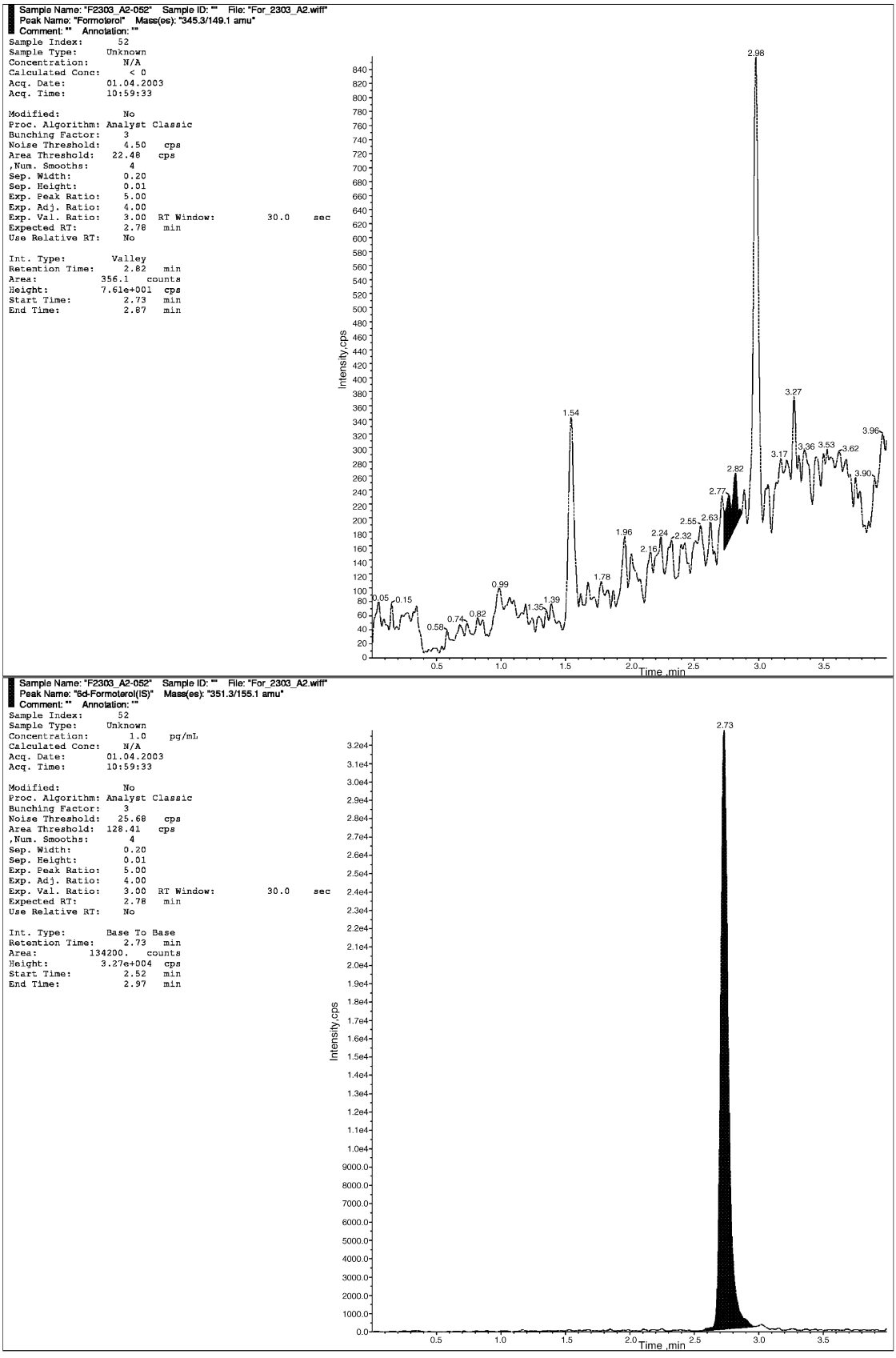


Fig. 3. Serum Standard 0 (Formoterol upper chromatogram; IS lower one).

were, at least in triplicate, incorporated into each sequence. According to the results of the QC samples a sequence was accepted or rejected. At least six of the nine QC-samples had to be within ±15% of their respective nominal values; three of the nine QC-samples (but not at the same concentration) may have been outside the ±15% of their respective nominal values.

If a batch did not adhere to these criteria, the batch was rejected. QC-samples outside ±15% (±20% at LLOQ level) are called "out of specifications", whereas QC-samples outside ±30% (±40% at LLOQ level) are called "outlier" (see Tables 3–6).

## 3. Results and discussion

Recent advances in the development of electrospray mass spectrometry made the specific detection of drugs in serum and plasma at low concentrations possible. Formoterol, a potent selective ß2-agonist, is inhaled in very low amounts of 9 μg. The latest generation of MS apparatus makes it possible to determine such low concentrations (absolute amounts of 20 fg Formoterol on column with about signal to noise of 5:1).

The other main problem when analysing Formoterol in serum or plasma samples is that there are several endogenous sub-

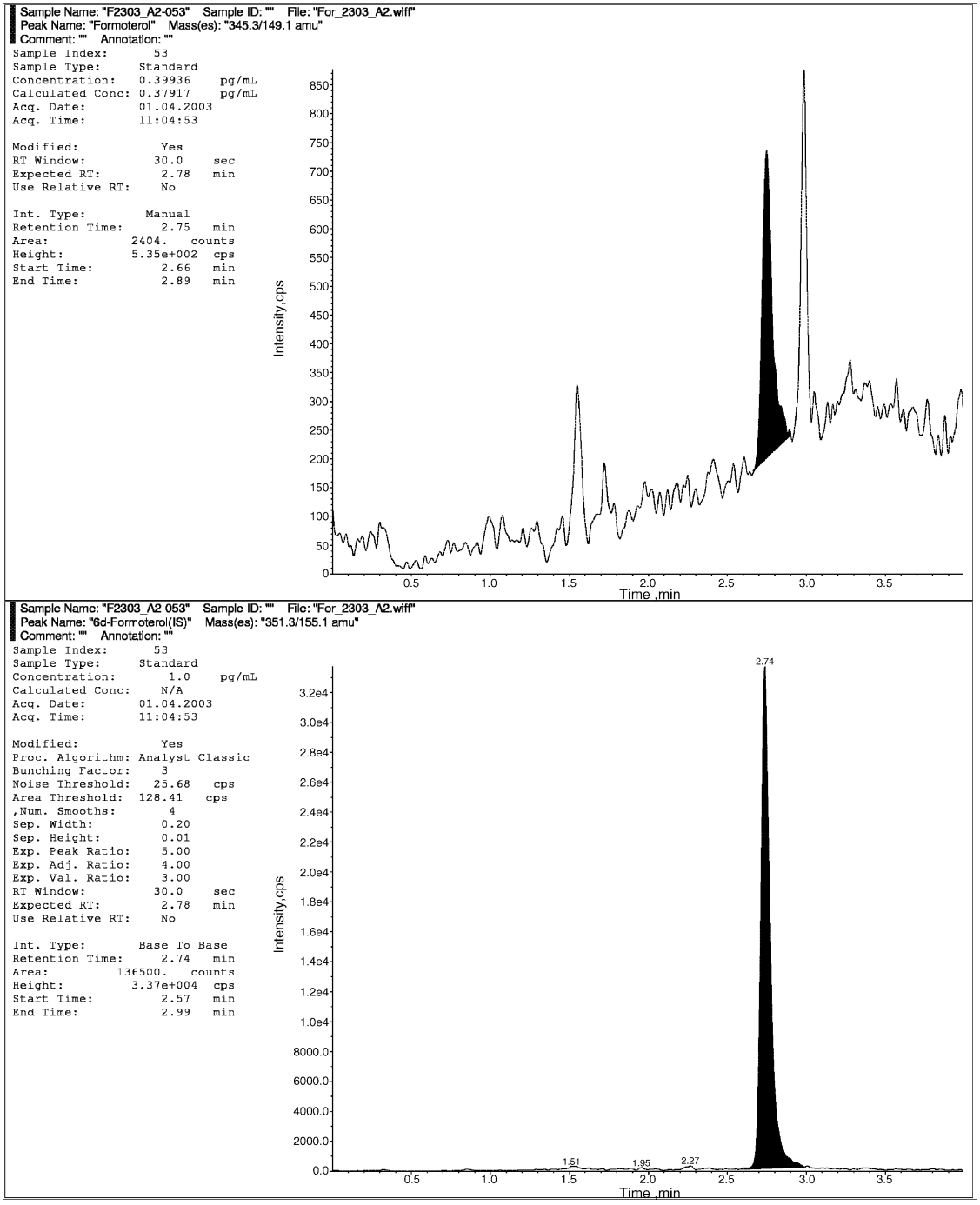


Fig. 4. Serum Standard 1; 0.40 pg/mL of Formoterol (upper chromatogram; IS lower one).

stances which can disturb and suppress the signal of the analyte at such low quantitation limits. Therefore, we tried to find a specific clean-up procedure with a high recovery rate for Formoterol. In our first attempt we tried liquid/liquid extraction but failed to extract the amphoteric molecule (amine and phenolic group). Clean-up with solid phase extraction (off-line with C18 cartridges or ENV + cartridges) caused problems regarding sufficient recovery (poor binding on RP material or insufficient elution). In addition, impurity in extracts caused matrix effects. Only solid phase extraction by SCX-3 (cation-exchange) with a highly specific clean-up procedure met the requirements. Such a specific clean-up is absolutely necessary when samples are very strongly enriched.

Finding a suitable internal standard was difficult (strong matrix effects) so at last a deuterated internal standard (d6) could be synthesized (TU Vienna) and purchased. It compensated fluctuations due to matrix effects.

The calibration curve was linear in the range from 0.40 to 100.24 pg/mL of Formoterol in human serum. Figs. 3–5 display chromatograms of calibration samples at different concentrations in human serum. Fig. 6 shows a calibration curve taken from batch one. The inter-batch precision of the cali-

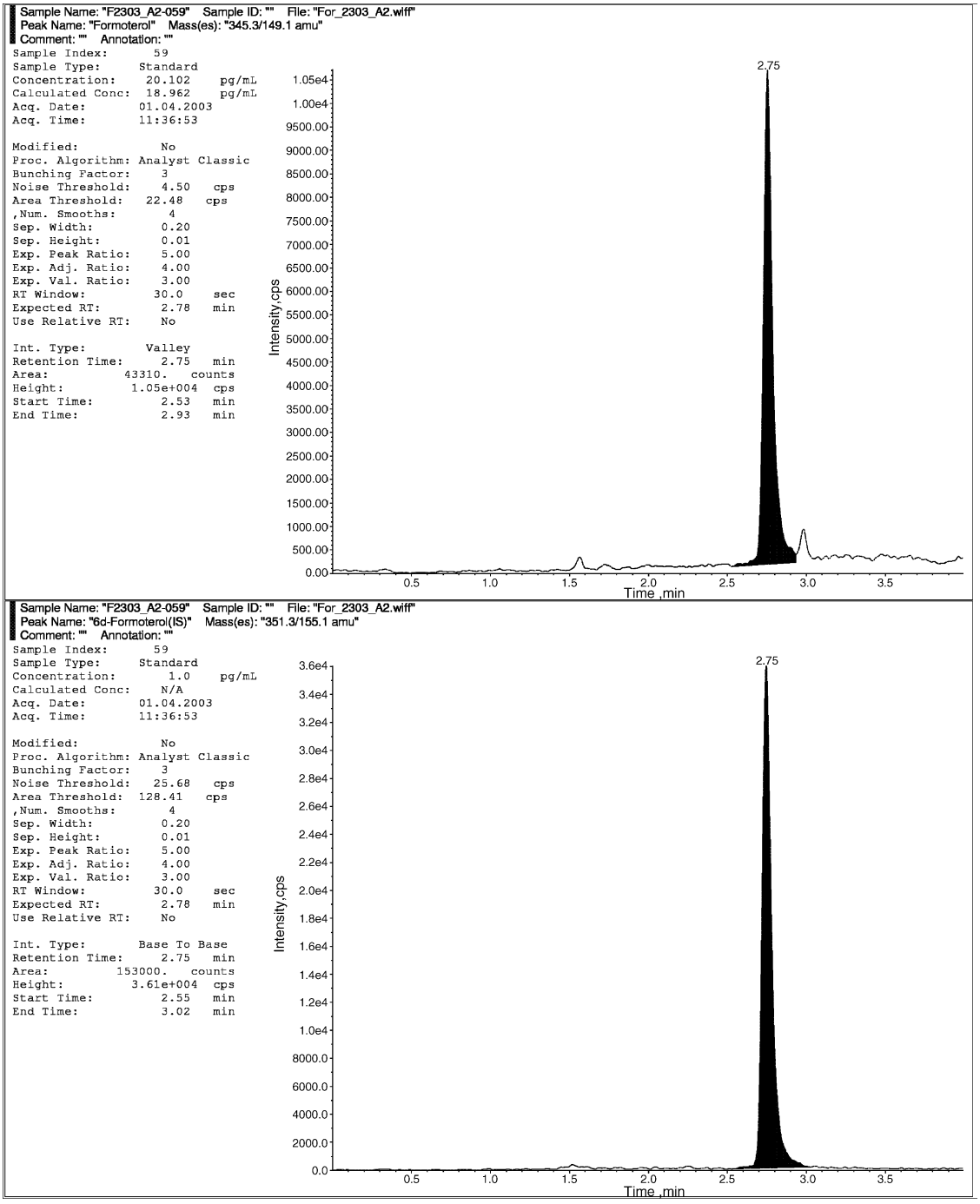


Fig. 5. Serum Standard 6; 20.10 pg/mL of Formoterol (upper chromatogram; IS lower one).

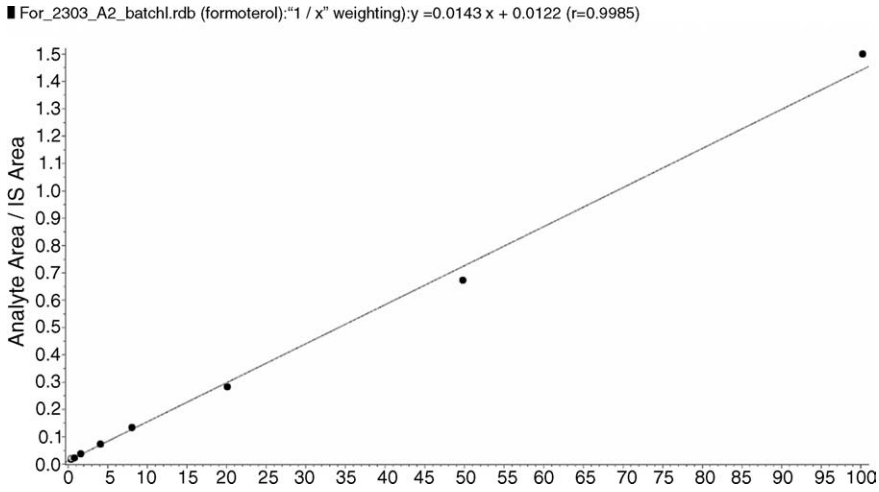


Fig. 6. Calibration curve of Formoterol from 0.40 to 100.24 pg/mL serum.

Table 1
Linearity, precision (CV) and accuracy of formoterol in human serum

| Sequence | Standard 1 | Standard 2 | Standard 3 | Standard 4 | Standard 5 | Standard 6 | Standard 7 | Standard 8 |
|---|---|---|---|---|---|---|---|---|
| Calculated concentration | | | | | | | | |
| For_B02303_A2 | 0.38 | 0.74 | 1.78 | 4.26 | 8.52 | 18.96 | 46.29 | 104.17 |
| For_B02303_A3 | 0.41 | 0.89 | 1.61 | 3.88 | 8.89 | 19.87 | 47.47 | 98.79 |
| | 0.40 | 0.75 | 1.49 | 3.94 | 8.26 | 20.34 | 45.74 | 107.45 |
| For_B02303_A4 | 0.40 | 0.76 | 1.48 | 4.24 | 8.94 | 20.33 | 46.51 | 102.43 |
| Mean | 0.40 | 0.78 | 1.59 | 4.08 | 8.65 | 19.88 | 46.50 | 103.21 |
| CV (%) | 3.32 | 9.01 | 8.81 | 4.84 | 3.73 | 3.26 | 1.55 | 3.50 |
| Expected concentration | 0.40 | 0.80 | 1.60 | 4.09 | 8.07 | 20.10 | 49.80 | 100.24 |
| Accuracy | 99.68 | 98.65 | 99.29 | 99.87 | 107.30 | 98.88 | 93.37 | 102.96 |

Table 2
Regression parameters of Formoterol in human serum

| Sequence | Intercept | Slope | *R* | Calculated range (pg/mL) | Number of standards | Weighting factor |
|---|---|---|---|---|---|---|
| For_B02303_A2 | 0.0122 | 0.0143 | 0.9985 | 0.40–100.24 | 8 | 1/*x* |
| For_B02303_A3 | 0.00816 | 0.0135 | 0.9984 | 0.40–100.24 | 16 | 1/*x* |
| For_B02303_A4 | 0.00552 | 0.0111 | 0.9989 | 0.40–100.24 | 8 | 1/*x* |

Table 3
Intra- and inter-batch precision (CV) and accuracy of Formoterol (Q-A)

| Sequence | Calculated concentration (pg/mL) | | | | | Mean (pg/mL) | CV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|
| | Value 1 | Value 2 | Value 3 | Value 4 | Value 5 | | | |
| Quality control sample Q-A (expected concentration 1.10 pg/mL) in human serum | | | | | | | | |
| For_B02303_A2 | 1.14 | 1.08 | 1.21 | 0.99 | 0.87 | 1.06 | 12.50 | 96.08 |
| For_B02303_A3 | 1.15 | 1.19 | [0.76] | 1.02 | 0.98 | 1.08 | 9.25 | 98.44 |
| For_B02303_A4 | 1.02 | 0.97 | 1.05 | 1.14 | 1.10 | 1.06 | 6.45 | 96.06 |
| Acceptable range (85–115%): 0.94–1.27 pg/mL | | | | | | | | |
| Inter-batch precision and accuracy (reproducibility), $n = 3$ batches | | | | | | | | |
| Mean (pg/mL) | 1.06 | | | | | | | |
| CV (%) | 9.05 | | | | | | | |
| Number | 14 | | | | | | | |
| Expected concentration (pg/mL) | 1.10 | | | | | | | |
| Accuracy (%) | 96.75 | | | | | | | |

*Note*: If outliers of QC-samples occurred, they were excluded from calculation and are displayed in brackets (e.g. [111]). Whereas QC-samples out of specification were included into calculation and are displayed as underlined values (e.g. 222).

Table 4
Intra- and inter-batch precision (CV) and accuracy of Formoterol (Q-B)

| Sequence | Calculated concentration (pg/mL) | | | | | Mean (pg/mL) | CV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|
| | Value 1 | Value 2 | Value 3 | Value 4 | Value 5 | | | |
| Quality control sample Q-B (expected concentration 6.00 pg/mL) in human serum | | | | | | | | |
| For_B02303_A2 | 6.34 | 5.80 | 6.13 | 6.44 | 6.15 | 6.17 | 4.01 | 102.82 |
| For_B02303_A3 | 6.77 | 6.06 | 6.63 | 5.95 | 6.57 | 6.40 | 5.75 | 106.57 |
| For_B02303_A4 | 6.45 | 6.94 | 6.70 | 6.67 | 6.11 | 6.57 | 4.75 | 109.52 |
| Acceptable range (85–115%): 5.10–6.90 pg/mL | | | | | | | | |
| Inter-batch precision and accuracy (reproducibility), $n = 3$ batches | | | | | | | | |
| Mean (pg/mL) | 6.38 | | | | | | | |
| CV (%) | 5.27 | | | | | | | |
| Number | 15 | | | | | | | |
| Expected concentration (pg/mL) | 6.00 | | | | | | | |
| Accuracy (%) | 106.3038 | | | | | | | |

*Note*: QC-samples out of specification were included into calculation and are displayed as underlined values (e.g. 222).

Table 5
Intra- and inter-batch precision (CV) and accuracy of Formoterol (Q-C)

| Sequence | Calculated concentration (pg/mL) | | | | | Mean (pg/mL) | CV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|
| | Value 1 | Value 2 | Value 3 | Value 4 | Value 5 | | | |
| Quality control sample Q-C (expected concentration 84.86 pg/mL) in human serum | | | | | | | | |
| For_B02303_A2 | 87.42 | 91.33 | 90.74 | 90.99 | 91.21 | 90.34 | 1.82 | 106.46 |
| For_B02303_A3 | 89.33 | 92.88 | 92.21 | 95.21 | 90.92 | 92.11 | 2.39 | 108.55 |
| For_B02303_A4 | 95.02 | 93.05 | 93.97 | 93.83 | 95.48 | 94.27 | 1.03 | 111.09 |
| Acceptable range (85–115%): 72.13–97.58 pg/mL | | | | | | | | |
| Inter-batch precision and accuracy (reproducibility), $n = 3$ batches | | | | | | | | |
| Mean (pg/mL) | 92.24 | | | | | | | |
| CV (%) | 2.47 | | | | | | | |
| Number | 15 | | | | | | | |
| Expected concentration (pg/mL) | 84.86 | | | | | | | |
| Accuracy (%) | 108.70 | | | | | | | |

bration standards in human serum for Formoterol ranged from 1.55% to 9.01%. The inter-batch accuracy (with reference to the mean value) of the calibration standards ranged from 93.37% to 107.30% for Formoterol (see Table 1). The linear regression parameters are displayed in Table 2. The intra-batch precision (CV) of the quality control samples in human serum for Formoterol ranged from 1.03% to 12.50%. The intra-batch accuracy (with reference to the mean value) of the quality control samples in human serum ranged from 96.06% to 111.09% for Formoterol (see Tables 3–5).

The inter-batch precision (CV) of the quality control samples in human serum for Formoterol ranged from 2.47% to 9.05%. The inter-batch accuracy (with reference to the mean value) of the quality control samples in human serum for Formoterol ranged from 96.75% to 108.70% (see Tables 3–5).

The intra-batch precision (CV) of the LLOQ samples in human serum was 19.67% for Formoterol. The intra-batch accuracy (with reference to the mean value) of the LLOQ samples in human serum was 96.78% for Formoterol (Table 6).

Several thousand of human serum and plasma samples (cross validation to human serum was successfully performed as well) deriving from clinical studies have been already analysed with this method (Tables 7 and 8).

Table 6
Lower limit of quantitation for Formoterol in human serum

| Sample | File name | Calculated concentration (pg/mL) | Mean (pg/mL) | CV (%) | Experimental concentration (pg/mL) | Accuracy (%) | Accuracy ref mean |
|---|---|---|---|---|---|---|---|
| LLOQ2 | F2303_A4-018 | 0.27 | 0.39 | 19.67 | 0.40 | 66.78 | 96.78 |
| LLOQ2 | F2303_A4-019 | 0.46 | | | 0.40 | 114.47 | |
| LLOQ2 | F2303_A4-020 | 0.43 | | | 0.40 | 108.38 | |
| LLOQ2 | F2303_A4-021 | 0.42 | | | 0.40 | 104.26 | |
| LLOQ2 | F2303_A4-022 | 0.36 | | | 0.40 | 90.00 | |
| LLOQ2 | F2303_A4-023 | 0.35 | | | 0.40 | 87.71 | |

*Note*: QC-samples out of specification were included into calculation and are displayed as underlined values (e.g. 222).

Table 7
Cross validation of Formoterol in human plasma

| Sample | File name | Calculated concentration (pg/mL) | Mean (pg/mL) | CV (%) | Experimental concentration (pg/mL) | Accuracy (%) | Accuracy ref mean |
|---|---|---|---|---|---|---|---|
| QC-AH | F3304_A2-045 | 1.22 | 1.09 | 12.10 | 1.10 | 110.7 | 99.4 |
| | F3304_A2-046 | 1.23 | | | 1.10 | 111.9 | |
| | F3304_A2-047 | 1.10 | | | 1.10 | 99.7 | |
| | F3304_A2-048 | 0.97 | | | 1.10 | 88.0 | |
| | F3304_A2-049 | 0.95 | | | 1.10 | 86.6 | |
| QC-BH | F3304_A2-064 | 5.92 | 5.79 | 3.49 | 6.00 | 98.8 | 96.6 |
| | F3304_A2-065 | 5.87 | | | 6.00 | 98.0 | |
| | F3304_A2-066 | 5.82 | | | 6.00 | 97.1 | |
| | F3304_A2-067 | 5.91 | | | 6.00 | 98.7 | |
| | F3304_A2-068 | 5.44 | | | 6.00 | 90.7 | |
| QC-CH | F3304_A2-078 | 86.47 | 87.49 | 1.03 | 85.15 | 101.5 | 102.8 |
| | F3304_A2-079 | 88.21 | | | 85.15 | 103.6 | |
| | F3304_A2-080 | 88.02 | | | 85.15 | 103.4 | |
| | F3304_A2-081 | 88.21 | | | 85.15 | 103.6 | |
| | F3304_A2-082 | 86.56 | | | 85.15 | 101.7 | |

Table 8
Lower limit of quantitation for Formoterol in human plasma

| Sample | File name | Calculated concentration (pg/mL) | Mean (pg/mL) | CV (%) | Experimental concentration (pg/mL) | Accuracy (%) | Accuracy ref mean |
|---|---|---|---|---|---|---|---|
| LLOQ-H | F3304_A2-025 | 0.48 | 0.45 | 4.73 | 0.40 | 119.8 | 112.6 |
| | F3304_A2-026 | 0.47 | | | 0.40 | 117.2 | |
| | F3304_A2-027 | 0.44 | | | 0.40 | 108.7 | |
| | F3304_A2-028 | 0.44 | | | 0.40 | 109.2 | |
| | F3304_A2-029 | 0.43 | | | 0.40 | 106.4 | |
| | F3304_A2-030 | 0.46 | | | 0.40 | 114.6 | |

Formoterol is stable in human serum and plasma below −20 °C for at least 5 months; further investigations regarding stability will be performed.

Formoterol is stable for at least 1 h in serum and plasma maintained at room temperature (about 25 °C). Three freeze/thaw cycles can be performed as long as thawed samples are frozen immediately (below −60 °C).

Samples may be frozen in injection solution before analysis. Formoterol was at least 24 h stable under auto sampler conditions.

The criterion for all stability measurements was 85–115% for accuracies.

Recovery was found to be 55.0% for Formoterol in human serum (determined at concentration levels of quality control samples) and 58.6% for the internal standard.

### 3.1. Examples of pharmacokinetic figures

A representative pharmacokinetic profile of Formoterol in human serum after the single inhalative administration of 9, 18 or 36 μg of Diformoterol fumarate dihydrate via drug powder inhaler is shown for one volunteer (subject 20) in Fig. 7 (log/lin diagram). This profile was taken from a study with 30 subjects.

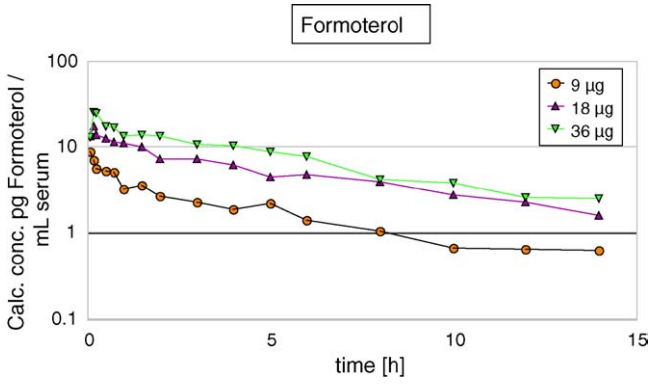


Fig. 7. Formoterol in human serum of one volunteer (subject 20) after inhalative application of 9–36 μg of Formoterol as Diformoterol-fumarate.

## 4. Conclusion

Quantitation of samples deriving from pharmacokinetic studies after inhalative administration of 9–36 μg Formoterol required a more sensitive method (below 1 pg/mL serum) than known from the literature [1] in order to calculate reliable pharmacokinetic characteristics. Our results show that Formoterol can be quantitated with a LLOQ of 0.40 pg/mL in serum or

plasma which is 10 times more sensitive than presented in the literature [1]. This has been achieved by the combination of a very efficient and selective sample clean-up with an ultra sensitive MS/MS instrument (API 4000).

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