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Journal of Chromatography B, 704 (1997) 221–229

JOURNAL OF CHROMATOGRAPHY B

Automated and sensitive method for the determination of formoterol in human plasma by high-performance liquid chromatography and electrochemical detection

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Received 2 April 1997; received in revised form 7 August 1997; accepted 21 August 1997

Abstract

An automated high-performance liquid chromatography (HPLC) method for the determination of formoterol in human plasma with improved sensitivity has been developed and validated. Formoterol and CGP 47086, the internal standard, were extracted from plasma (1 ml) using a cation-exchange solid-phase extraction (SPE) cartridge. The compounds were eluted with pH 6 buffer solution–methanol (70:30, v/v) and the eluate was further diluted with water. An aliquot of the extract solution was injected and analyzed by HPLC. The extraction, dilution, injection and chromatographic analysis were combined and automated using the automate (ASPEC) system. The chromatographic separations were achieved on a 5 μm, Hypersil ODS analytical column (200 mm×3 mm I.D.), using (pH 6 phosphate buffer, 0.035 *M*+20 mg/l EDTA)–MeOH–CH_3CN (70:25:5, v/v/v) as the mobile phase at a flow-rate of 0.4 ml/min. The analytes were detected with electrochemical detection at an operating potential of +0.63 V. Intra-day accuracy and precision were assessed from the relative recoveries of calibration/quality control plasma samples in the concentration range of 7.14 to 238 pmol/l of formoterol base. The accuracy over the entire concentration range varied from 81 to 105%, and the precision (C.V.) ranged from 3 to 14%. Inter-day accuracy and precision were assessed in the concentration range of 11.9 to 238 pmol/l of formoterol base in plasma. The accuracy over the entire concentration range varied from 98 to 109%, and precision ranged from 8 to 19%. At the limit of quantitation (LOQ) of 11.9 pmol/l for inter-day measurements, the recovery value was 109% and C.V. was 19%. As shown from intra-day accuracy and precision results, favorable conditions (a newly used column, a newly washed detector cell and moderate residual cell current level) allowed us to reach a LOQ of 7.14 pmol/l of formoterol base (3 pg/ml of formoterol fumarate dihydrate). Improvement of the limit of detection by a factor of about 10 was reached as compared to the previously described methods. The method has been applied for quantifying formoterol in plasma after 120 μg drug inhalation to volunteers. Formoterol was still measurable at 24 h post-dosing in most subjects and a slow elimination of formoterol from plasma beyond 6–8 h after inhalation was demonstrated for the first time thanks to the sensitivity of the method.

Keywords: Formoterol

1. Introduction

Formoterol is a potent selective β_2-adrenoceptor agonist formulated as the fumarate [1]. The data on the pharmacokinetics of formoterol in plasma or blood of humans are limited [2,3] due to the very low concentrations (in the low pmol/l range) reached

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0378-4347/97/$17.00
PII S0378-4347(97)00425-8

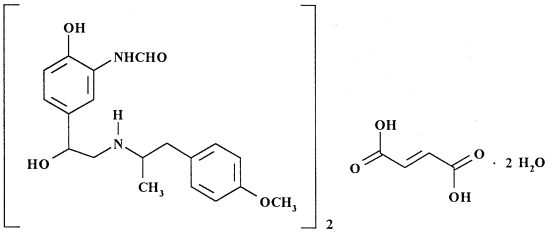
after therapeutic doses and the problems arising therefrom, i.e., to develop an analytical method sensitive enough to determine these concentrations.

A high-performance liquid chromatography (HPLC) method involving electrochemical detection has been described by van den Berg et al. [2] for the determination of formoterol in plasma. This assay achieved a sensitivity (limit of detection: 20 pg/ml, i.e., about 50 pmol/l of formoterol base by using 2-ml plasma samples) which was not attainable with any previous methods [4,5]. The approach used by the authors appeared very appropriate for the physiochemical properties of formoterol. Selective cation-exchange solid-phase extraction (SPE), HPLC and electrochemical detection at a rather low potential of +0.63 V to allow selective oxidation were combined. Despite the low detection limit reached with this method, it was not sensitive enough to determine formoterol in plasma at therapeutic levels.

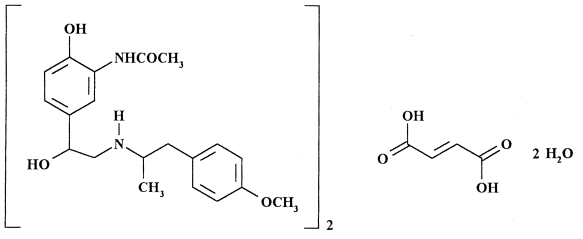
The objective of the present work was to develop and validate a quantitative method for formoterol in human plasma with a sensitivity higher than that obtained by van den Berg. This previous HPLC method [2] was used as an initial basis for the development.

The chemical structures of formoterol and CGP 47086A, the internal standard (I.S.), are as follows:

Formoterol fumarate dihydrate (active drug substance)



CGP 47086A (internal standard)



Unless otherwise mentioned, the concentration values in the report are expressed in molar units of formoterol base (1 pmol/l of formoterol base corresponds to 0.3444 pg/ml of formoterol base and to 0.4205 pg/ml of formoterol fumarate dihydrate).

2. Experimental

2.1. Chemicals and reagents

Formoterol fumarate dihydrate and CGP 47086A were supplied by Ciba-Geigy (Basle, Switzerland). All solvents and reagents referred below were of analytical grade and used without further purification: acetonitrile (Ref. RS412412), methanol (Ref. RS 525102 for the mobile phase and Ref. RS-ACS 414902 for the liquid–solid extraction) from Carlo-Erba France (Nanterre, France), potassium dihydrogenphosphate (Ref. 1.04873), anhydrous di-sodium hydrogenphosphate (Ref. 6586) from Merck (Darmstadt, Germany), potassium chloride (Ref. P/4280/53) from Fisons (Loughborough, UK), ethylenediaminetetraacetic acid (EDTA) (Ref. 25 235-2) from Aldrich (Dorset, UK), water from Mallinckrodt Backer (Deventer, Netherlands) and (SPE) cartridges of 1 ml capacity containing 100 mg polysulfonic (PRS) sorbent (strong cation-exchange), Bond-Elut (Ref. 1210-2012) from Varian and supplied by Prolabo (Paris, France).

Phosphate solutions: pH 6.6 phosphate buffer for sample dilution: 0.05 *M* KH_2PO_4–0.05 *M* Na_2HPO_4 (62.7:37.3, v/v). Diluted (0.025 *M*) pH 6.6 phosphate buffer for cartridge conditioning and washing. Elution solution on SPE cartridge: pH 6 buffer–methanol (70:30, v/v). The pH 6 buffer consisted of [0.03 *M* KH_2PO_4–0.03 *M* Na_2HPO_4 (87.7:12.3, v/v)]+1.5 g of KCl for 100 ml preparation.

Mobile phase: acetonitrile–methanol–pH 6 buffer solution (5:25:70, v/v/v). The buffer solution consisted of [0.035 *M* KH_2PO_4–0.035 *M* Na_2HPO_4 (87.7:12.3, v/v)]+40 mg of EDTA for a 2-l preparation.

2.2. Standard solutions and samples

The stock solution of formoterol was prepared by dissolving 1 mg of the test substance (formoterol

fumarate dihydrate) in 10 ml of methanol. A 1 ml volume of this stock solution was further diluted to 100 ml with methanol. Appropriate serial dilutions of the diluted stock solution with water were then made in order to prepare the spiking solutions at concentrations ranging from 60 pg/ml to 2.5 ng/ml formoterol fumarate dihydrate. The spiking solutions were used for the preparation of the calibration samples. Another stock solution of formoterol at the same concentration was prepared in the same conditions from a second weighing and appropriately diluted to give spiking solutions to be used for preparation of validation (accuracy and precision assessments), quality control and stability samples. The I.S. stock solution was prepared by dissolving 0.1 mg of CGP 47086A in 10 ml of methanol. A 1 ml volume of I.S. stock solution was further diluted up to 10 ml with methanol. A 1 ml volume of the diluted I.S. stock solution was further diluted up to 200 ml with water resulting in the internal standard spiking solution (5 ng/ml). All the solutions were prepared in glass flasks and stored at about +4°C while not in use.

Standard/calibration samples in the concentration range of 7.14 to 297 pmol/l of formoterol base (3 to 125 pg/ml of formoterol fumarate dihydrate) were prepared for calibration, accuracy and precision, quality control and stability assessments by adding 50 μl of appropriate spiking solutions to 1 ml of drug-free human plasma aliquots. The samples were analyzed as described in Section 2.4.

2.3. Instrumentation and chromatography

The instrumentation consisted of a pump, Model LC-10AD from Shimadzu (Kyoto, Japan), an electronic degasing system, Model Degasys DG 1310, from Uniflow, supplied by Touzart et Matignon (Les Ulis, France), an automate system/autosampler, Model XL, ASPEC (automatic sample preparation with extraction columns) from Gilson (Villiers le Bel, France) with refrigerated rack at 3°C, an electrochemical detection system, Model Decade from Antec (Leiden, Netherlands) equipped with a pulse damper and a VT-03 flowcell using a working electrode with a 50 μm thickness spacer at an operating potential of +0.63 V in DC mode, range 0.2 nA full scale connected in recorder output mode, 5 s noise filter. The oven of the detector allowed to maintain both the cell and the HPLC column at 33°C. Data acquisition was operated from a workstation, Millennium 2.1 from Waters (Milford, MA, USA).

The chromatographic separations were performed at 33°C on a 5 μm particle Hypersil ODS analytical glass column (200 mm long as two directly connected 100 mm long cartridges×3 mm I.D.) (Ref. 27760) supplied by Chrompack France (Les Ulis, France). The analytical column was protected with a reversed-phase R2 Chromsep guard column (10 mm×2 mm I.D.) (Ref. 28141), supplied by Chrompack France. The mobile phase was delivered at a flow-rate of 0.4 ml/min. Its preparation is described in Section 2.1. It was not filtered before use. The connections were made by using PEEK (polyether ether ketone) tubing, 0.02 in. I.D. from the pump to the injector, 0.005 in. I.D. from the injector to the column and PTFE tubing from the column output to the detector input and from the detector output to waste (1 in.=2.54 cm).

Conditioning of the analytical and guard columns, i.e., washing for about four days with the mobile phase, is necessary before connecting them to the detector cell.

2.4. Sample preparation

To 1 ml of plasma in a polypropylene tube were successively added, a 50 μl aliquot of either appropriate spiking solution for validation, calibration/standard samples (see Section 2.2) or of water for actual/unknown samples, 50 μl of I.S. spiking solution and 0.75 ml of pH 6.6 buffer solution, 0.05 *M*. The tube was then vortexed at high speed for about 5 s and placed on the refrigerated rack of the automate. The sample preparation was then performed by the automate according to the flow chart in Table 1. After each transfer of liquid, the needle of the automate was rinsed with 1 ml of EDTA in water 20 mg/l–methanol (90:10, v/v). Each sample was prepared during the chromatography of the previous sample. To avoid an increase of the column pressure and a frequent replacement of the guard column, the diluted extract before the injection was filtered on the ASPEC system using a nylon filter (0.45 μm) set at the bottom of an empty cartridge.

Table 1
Automate flow-chart for sample preparation

Steps	Fluid	Aspire flow-rate (ml/min)	Dispense[a] flow-rate (ml/min)
Condition the cartridge	1. 2 ml CH_3OH	3	6
	2. 0.5 ml water	6	6
	3. 3 ml pH 6.6 phosphate buffer, 0.025 *M*	6	3
	4. 1 ml water	6	3
Load the sample	1.85 ml sample solution	3	0.5
Wash	1. 2.5 ml pH 6.6 phosphate buffer, 0.025 *M*	6	1.5
	2. 0.5 ml water	6	1.5
	3. 0.25 ml water–CH_3OH (80:20, v/v)	3	3
Dry	2 ml air	3	6
Elute	150 μl pH 6 buffer–CH_3OH (70:30, v/v)	3	3
	150 μl pH 6 buffer–CH_3OH (70:30, v/v)	3	1.5
Dry	1 ml air	3	6
Dilute the extract	0.2 ml water	3	6
Mix	1 ml air	6	12
Optional filtration: Rinse the cartridge and the filter	1 ml pH 6 buffer–CH_3OH, (70:30, v/v)	3	3
	4.5 ml air	3	3
Load the diluted extract	0.5 ml diluted extract	3	3
Dry	4.5 ml air	3	3
Inject (300 μl loop)	400 μl diluted extract	–	1.5

[a] No air push volume is used for dispensing.

2.5. Calibration and sample quantitation

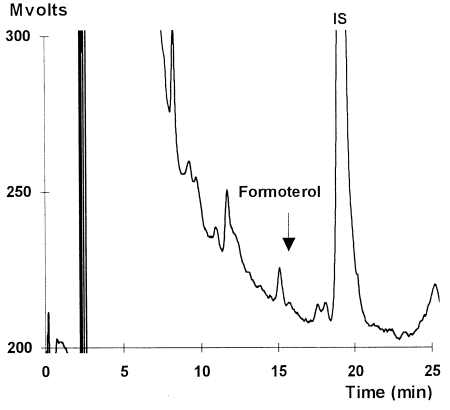
Calibration standard samples at six or seven different concentrations in single in the range 7.14 to 297 pmol/l of formoterol were prepared as described in Section 2.2 and analyzed as described in Sections 2.3 and 2.4. Calibration curves ($y=mx+b$), represented by the plots of the peak height ratios (y) of formoterol base to I.S. versus the concentrations (x) of the calibration samples, were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model [6]. Concentrations in clinical/preclinical, quality control and stability samples were calculated from the resulting peak height ratios and the regression equation of the calibration curve.

3. Results and discussion

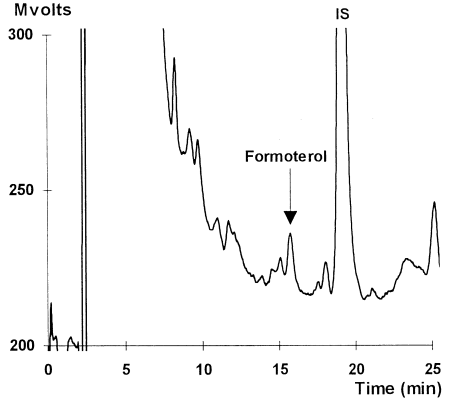
3.1. Chromatography/specificity

Representative chromatograms of extracts of human plasma are shown in Fig. 1. Formoterol and I.S. were eluted from the analytical column with retention times of approximately 15.6 and 19.0 min, respectively. The compounds of interest were separated from co-extracted endogenous plasma components for four different plasmas from volunteers and rats not given any medication. Three analytical columns with the same reference as defined in Section 2.3. demonstrated comparable chromatographic characteristics. The chromatogram for an

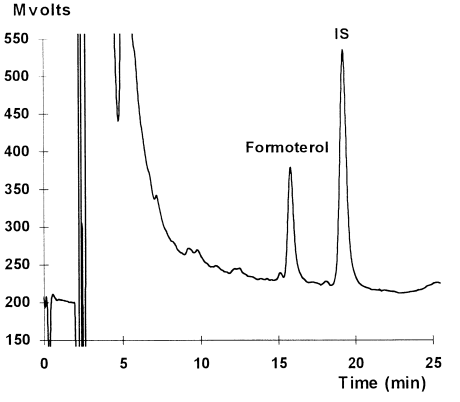
A: Drug-free plasma sample, with site of formoterol and spiked with 575 pmol/L CGP 47086 (IS). Signal units: 1000 Mvolts = 0.2 nA



B: Plasma sample spiked with 23.8 pmol/L of formoterol and 575pmol/L of IS.



C: Plasma sample spiked with 238 pmol/L of formoterol and 575 pmol/L of IS.



D: Plasma sample from a volunteer taken 8 h after a 120 μg inhaled dose of formoterol, spiked with CGP 47086A. Found formoterol concentration: 32.8 pmol/L

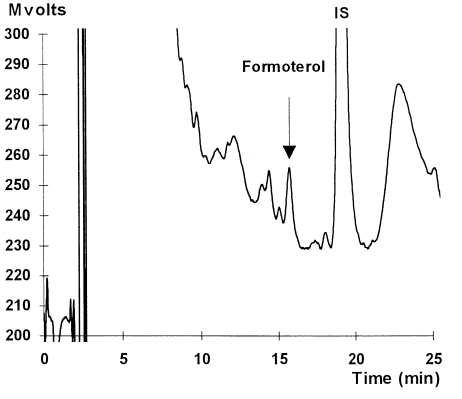


Fig. 1. Formoterol in human plasma: representative chromatograms.

extract of a sample from one volunteer after inhalation of a 120 μg dose of formoterol (Fig. 1D) was similar to that of standard samples.

3.1.1. Electrochemical detection

Due to the extreme sensitivity requested, special care should be taken to prevent electrochemical contamination in the cell of the detector. Such contamination could last for several days and leads to high residual cell current level (I_{cell}), passivation of the detector cell and sensitivity decrease. The purity of the mobile phase components and the conditioning of analytical and guard columns appeared particularly critical. Glass tubing HPLC columns proved to be more rapidly conditioned than the stainless-steel ones. Typically, for the glass column, about four days of conditioning by mobile phase circulation at a 0.3 ml/min flow-rate, are required before connecting it to the detector. I_{cell} level depends mainly on the column and the mobile phase characteristics. In the described conditions, the I_{cell} current should stabilize at a level smaller than 1 nA. An I_{cell} current stabilized at a level higher than 1 nA could indicate incorrect conditioning of the column/pre-column or a contamination from the mobile phase. When a decrease of sensitivity was observed, washing the cell glassy carbon surface with acetone (30 min in ultrasonic bath) was sufficient to recover suitable sensitivity.

3.2. Extraction efficiency

The extraction efficiency of formoterol from plasma was assessed by comparison of the peak height from extracted samples (concentrations 238 and 47.6 pmol/l) to those from formoterol directly spiked to drug-free plasma extracts. The mean efficiencies of extraction were respectively 73% (238 pmol/l) and 78% (47.6 pmol/l). Using a similar procedure, the extraction efficiency of the I.S. was 74%. In agreement with previous findings [2], maximum recoveries were achieved at pH 6.6.

3.3. Calibration curves

The calibration curve statistics are shown in Tables 2 and 3. The correlation coefficients were higher than 0.999. Individual fit of the calibration standards to the curve was assessed from the relative error (R.E. in %): 100×[(regressed concentration calculated from the curve equation and the peak height ratio)(nominal concentration)]/(nominal concentration) [7]. As shown in Tables 2 and 3, the differences for regressed concentrations did not exceed 6% from theory and the sign of the R.E.% does not appear to be concentration dependent. This indicated a good fit of the regression model over the range of the calibration curve.

Table 2
Formoterol in plasma: calibration curve statistics

Analysis day	Slope	*y*-Intercept	Correlation coefficient (*r*)
27 June 1996	0.002105	0.001458	0.9992
01 July 1996	0.002163	0.005459	0.9991
11 July 1996	0.002094	0.005319	0.9996
Mean	0.002121	0.004079	0.9993
S.D.	0.000037	0.002271	0.0003

3.4. Accuracy and precision

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest, near the lowest, near the middle and the highest concentration values of the calibration range. Accuracy was determined by calculating the mean value for the found concentrations in % of the nominal concentrations in standard samples. Precision was assessed from the coefficient of variation (C.V.) of the mean concentrations. The following validation criteria for accuracy and precision were used to assess the method suitability: mean concentrations should be within 85–115% except at the limit of quantitation (LOQ) where it should be within 80–120%; C.V. should not exceed 15%, except at the LOQ where it should not exceed 20% [8].

3.4.1. Intra-day measurements

Samples were analyzed on the same day. Individual, mean concentrations and corresponding C.V.s are presented in Table 4. Mean concentrations ranged from 81% to 105% of the nominal values over the 7.14 to 238 pmol/l of formoterol base

Table 3
Formoterol in plasma: individual fit of the calibration samples to the curves

Nominal concentration (pmol/l)	Analysis day						Accuracy: mean measured concentration (% of nominal)	Precision: C.V. (%)
	27 June 1996		1 July 1996		11 July 1996			
	Measured concentration	R.E. (%)[a]	Measured concentration	R.E. (%)[a]	Measured concentration	R.E. (%)[a]		
7.14	7.32	2.52	7.08	−0.85	7.00	−2.02	100	2.3
11.9	11.7	−1.70	12.0	0.55	12.1	2.06	100	1.7
23.8	22.4	−5.82	24.8	4.18	24.6	3.19	101	5.6
47.6	48.2	1.28	45.2	−5.05	47.6	0.00	99	3.4
119	118	−1.23	120	0.45	116	−2.31	99	1.7
238	242	1.69	231	−3.10	232	−2.51	99	2.6
297	307	3.25	308	3.83	302	1.57	100	1.1

[a] R.E. (%)=100×[(measured concentration from the curve equation and y values)−(nominal concentration)]/(nominal concentration).

Table 4
Formoterol in plasma: intra- and inter-day accuracy and precision

Nominal/added concentration (pmol/l)	No. of values	Accuracy: mean found concentration (% of nominal)	Precision: C.V. (%)
Intra-day			
7.14	5	81	14
11.9	5	98	10
23.8	6	93	9
119	6	94	6
238	6	105	3
Inter-day[a]			
11.9	18	109	19
23.8	58	99	15
47.6	68	100	12
119	39	98	8
238	69	101	9

[a] Samples were analyzed on 33 different days over a period of two months.

concentration range, with the C.V. ranging from 3 to 14%.

3.4.2. Inter-day measurements

Samples were analyzed on 33 different days over a period of two months. Mean concentrations and C.V.s are presented in Table 4. Mean concentrations ranged from 98 to 109% of theory and C.V.s from 8 to 19% over the 11.9 to 238 pmol/l of formoterol base concentration range.

3.5. Limit of quantitation

The LOQ is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. As indicated earlier in this section, the mean concentration should be within 80–120% of the nominal value with a C.V. not exceeding 20%. The lowest concentration value of 11.9 pmol/l of formoterol base whose inter-day accuracy and precision (Table 4) were within the proposed criteria is quoted as the LOQ. Nevertheless, with a newly used column, a newly washed detector cell and moderate electrochemical background level, an intra-day LOQ of 7.14 pmol/l (3 pg/ml of formoterol fumarate dihydrate) (see Table 4) was reached. The limit of detection was about 4 pmol/l. An improvement of the limit of detection by a factor of about 10 was reached as compared to the previously described sensitive HPLC method [2].

3.6. Stability

3.6.1. Standard solutions

The standard solutions were found to be stable for at least three months at about +4°C: the concentrations after such storage conditions were 98% (475 pmol/l) and 112% (4.75 nmol/l) for formoterol base and 99% for I.S. solution of the concentration values found with freshly prepared solutions.

3.6.2. Blood samples

Formoterol was found to be stable in blood samples kept for at least 15 min at 37°C.

3.6.3. Effect of freeze–thaw cycles

Standard plasma samples, in duplicate, with concentrations near the lower and upper limits of the calibration curve were prepared and immediately frozen at about −20°C. The samples were thawed and analyzed according to the procedure in Section 2.4. No loss was observed after three freeze–thaw cycles.

3.6.4. Sample analysis

The plasma samples diluted with pH 6.6 buffer were found to be stable for at least 15 h at +3°C on

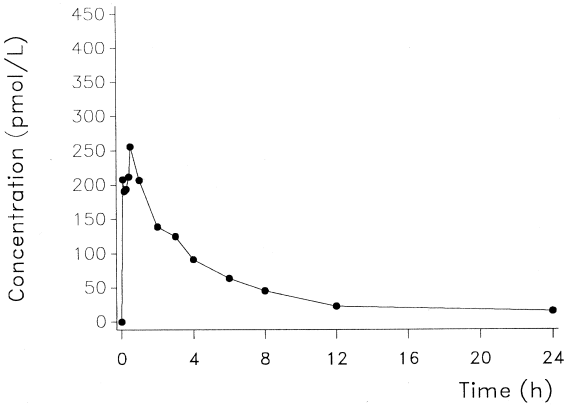


Fig. 2. Formoterol base concentration–time curve in plasma after formoterol fumarate dry powder inhalation (dose: 120 μg) to a healthy volunteer.

the sample tray of the automate/injection system. The concentrations following this storage period were 100 and 98% of the nominal values of 23.8 and 238 pmol/l, respectively.

3.7. Application

The method was applied to plasma samples from twelve healthy volunteers after inhalation of a 120 μg single dose of formoterol fumarate. A curve of concentration of formoterol base in plasma of one volunteer is exemplified in Fig. 2. The concentration measured at 24 h in this subject, 15.3 pmol/l, is similar to the global mean concentration of the twelve subjects.

4. Conclusions

An automated and sensitive method is available and has been applied for quantifying formoterol in human plasma after 120 μg drug inhalation. Formoterol was still measurable at 24 h post-dosing in most subjects and a slow elimination of formoterol from plasma beyond 6–8 h after inhalation was demonstrated for the first time thanks to the sensitivity of the method.

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