Original Article

Method Development and Validation for Detection of Procaterol in Human Urine using Gas Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Background: A simple, sensitive and accurate method for the determination of procaterol in human urine was developed and validated using Gas Chromatography coupled to tandem mass spectrometry (GC-MS/MS) as per the requirements of World Anti-Doping Agency (WADA) and ICH guidelines. **Methodology:** The sample processing includes deconjugation with enzymatic hydrolysis, Solid Phase Extraction (SPE) procedure using XAD2 column and Liquid-liquid Extraction (LLE) followed by the derivatisation using N-methyl trimethylsilysl trifluoroacetamide (MSTFA). **Results:** The method included a chromatographic run of 17 min and the calibration curve was linear over the concentration range of 5-40 ng/mL with a correlation coefficient (r) of 0.98. The intra- and inter-day assay precision ranged from 0.75 to 0.94% and 1.2 to 1.70%, respectively. The absolute recovery percentage for procaterol was found to be 83%. The recovery was sufficient to reliable identify the procaterol at or below the level prescribed by WADA ie MRPL 20ng/ml. The LOQ and LOD was found to be 5 and 3 ng/ml respectively. **Conclusion:** A simple and sensitive GC-MS/MS method for quantitative estimation of Procaterol was developed and validated as per the WADA ISL and ICH guidelines. This developed method could be also used for therapeutic drug monitoring purposes.

Key words: Procaterol, Validation, ICH Guideline, GC-MS/MS, Doping control, WADA.

INTRODUCTION

Elite athletes are more susceptible to developing asthma; therefore, the prevalence of asthma and exercise-induced bronchoconstriction is higher than in the background population, making β_2-agonists among the most used drugs in competitive sport.[1-3] It is well known that β_2-Adrenoceptor agonists are a useful therapy for asthma. These drugs also exhibit an anabolic effect due to an increase in β_2-adrenoceptor-mediated protein accretion, thus their use by athletes is prohibited by the World Anti-Doping Agency (WADA). WADA code has declared that one should ensure that no prohibited substances should enter in the body and the medication taken should be used in case of illness producing valid Therapeutic use Exemption certificate (TUE) and that the general safety of the athletes is of utmost importance.[3-5]

In competitive sports, salbutamol, salmeterol and formoterol are now allowed without a Therapeutic Use Exemption (TUE) and clinical proof of asthma or exercise-induced bronchoconstriction, while other β_2-agonists are allowed if the athlete acquires a TUE. To acquire a TUE, the athlete must meet different medical criteria and have a positive bronchial challenge or reversibility test to β_2-agonists. One of the β_2-agonists that require a TUE is procaterol, which is a commonly prescribed β_2-agonist in Asia and southern Europe.[1,2]

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Procaterol is a potent, fast-acting β_2-agonist with a 2(1H)-quinolinone chemical skeleton as depicted in Figure 1. It has a long duration of action, used in treatment of asthma by oral or inhalation route. Because of its high intrinsic efficacy and high selectivity for bronchial smooth muscle, procaterol is effective in microgram doses.[6] However, given the low doses of used procaterol, detection and quantification is difficult in serum and urine.[1,6,7]

In 1980, no sensitive method was available for detection of Procaterol but since 2010, a sensitive and reproducing method was developed which is High performance Liquid Chromatography-Tandem Mass Spectroscopy (HPLC-MS/MS). This method is mainly used for the determination of pharmacokinetic profile of oral Procaterol in urine. By using this method, the detection of parent Procaterol and its metabolite is possible in human urine and serum. The metabolite of Procaterol are measured upto 12 hr after administration but the parent Procaterol is measured upto 24 hr after administration.[1,8]

Several methods have been reported, suggesting separation of β_2-Agonist compounds by using different analytical methods for the determination of β_2-Agonists from biological fluids such as Liquid Chromatography-Mass Spectrometry (LC-MS),[1,2,9,10] Gas Chromatography- Mass Spectrometry (GC-MS)[11,12] and flow injection analysis coupled with electro generated chemiluminescence (FIA-ECL).[13]

Moreover, the procaterol is metabolized into two metabolites (DM-251 and DM-252) along with the parent drug. However, the metabolites are only measurable up to 12 hr after administration, whereas unchanged procaterol is detectable up to 24 hr after administration. Using this method, the only unchanged procaterol can be measured in urine. Gas Chromatography (GC) is a common and attractive method for the detection and analysis of β_2-Agonist and its analogs compounds, prior to injection a derivatization step is important which makes Gas Chromatography (GC) a more reliable and allow a routine determination and analysis of β_2-Agonist compounds in human urine samples. The purpose of the present study was thus to develop a selective and validated GC-MS/MS method for the determination of procaterol in human urine.

MATERIALS AND METHODS

Material, Chemical and Standard Solution

All reagents and chemicals used were of analytical grade or HPLC grade and purchased from different manufacturers. Methanol, Potassium dihydrogen phosphate and Potassium carbonate were obtained from Merck (Mumbai, India), Tertiary Butyl Methyl Ether (TBME) from JT Baker (Phillipburg, USA), β-Glucuronidase (*E. coli*) enzyme from Roche Diagnostics Corporation (Indiapolis, USA). XAD2 from Supelco, Acetone, Anhydrous Sodium Sulphate, MSTFA, Iodo-trimethylsilane and Dithioerythitol were obtained from Sigma Aldrich (Mumbai, India). The certified reference standards of steroids and/or their metabolites were obtained from established sources like Sigma-Aldrich, USA, National Measurement Institute, Australia, Cerilliant, USA. The standards Certified Reference Materials (Procaterol HCl and 17a-Methyl Testosterone) were purchased from sigma (Mumbai, India).

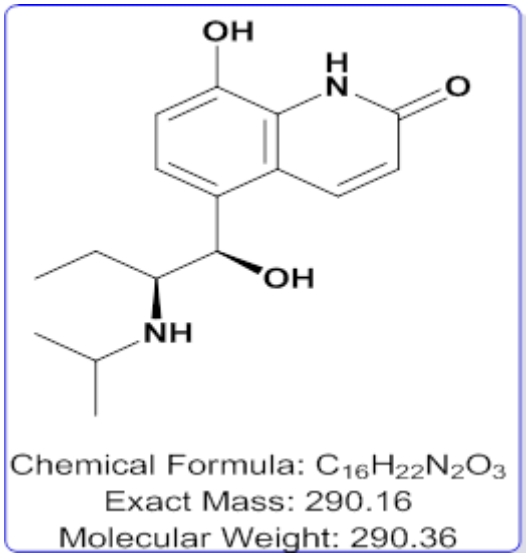

Figure 1: Molecular Structure of Procaterol.

Instrumentation and chromatographic conditions

GC-MS/MS Analysis was performed using Shimadzu QqQ Mass spectrometer TQ 8050, Shimadzu GC-2010 Plus equipped with AOC-20i Automatic Liquid Sampler (ALS). HP Ultra-1 (17m X 220µm X 0.11µm) was used to separate the analyte. Spectral detection was carried out on TQ 8050. The GC-MS/MS data were acquired and processed using GC Labs Solution and Insight GCMS Software.

The temperature program was as follows: the initial temperature was 100°C (0.2 min), increased at 90°C/min to 190°C (0.2), then at 10°C/min to 225°C and at 90°C/min to a final temperature of 310°C (held for 0.95 min). The transfer line was set at 280°C. Helium (Air Liquid) was used as a carrier gas at a flow rate of 0.8 mL/min followed by split mode at a ratio 11:1. The total run time of the method was found to be less than 17 min. The MS quadrupole temperature was kept at

150°C, while the MS source operated at 230°C in Electron Impact (EI) mode, with a solvent delay of 2 min. Data were acquired in the Multiple Reaction Monitoring (MRM) mode, by using this diagnostic transition of the analyte (Procaterol) as shown in Table 1.

Sample preparation

A 2 mL aliquot of urine was applied into the XAD2 column and spiked with 50 µL of the internal standard mixture. For hydrolysis samples were incubated in an oven at 60°C for 1 h after adding 1mL of 0.1 M phosphate buffer solution (pH=7) and 50 µL of b-glucuronidase. After adding 250 µL of 7% potassium carbonate solution (pH=9-10), the mixture was extracted for 10 min with 5mL Tert Butyl methyl ether (TBME) by using horizontal shaker. The organic phase was centrifuged for 5 min at approximately 2000 rpm, dried with Na_2SO_4 and evaporated under nitrogen at room temperature.

A derivatization procedure of the dried residue using 50 µl of MSTFA / IODO –TMS / DTE mixture (1000/2/2: v/v/w) for 30 min at 60°C was used to achieve optimum derivatization efficiency. A 2 µL aliquot of this mixture was injected into the GC for analysis.

The MS quadrupole temperature was kept at 150°C, while the MS source operated at 230°C in Electron Impact (EI) mode, with a solvent delay of 2 min. Data were acquired in the Multiple Reaction Monitoring (MRM) mode, by using this diagnostic transition of the analyte (Procaterol) as shown in Table 1.

Validation procedures

The method was validated in accordance with ICH guidelines and requirements of WADA ISL [24-25] To establish linearity and range, a stock solution containing 1 mg/ml drug in ethanol was diluted to yield solutions in the concentration range 5-40ng/ml and keeping the injection volume constant (2 µL). To assess precision, five injections of three different QC concentrations (8, 15, 35 ng/ml) were made on the same day and intra-day precision was determined as relative standard deviation. These studies were also repeated on different days to determine inter-day precision. Accuracy was evaluated by fortifying a mixture of decomposed reaction solutions with three known concentrations of the drug and recovery of the added drug was evaluated. The specificity of the method for the drug was established by analyzing human urine samples collected from ten different human to investigate the potential interferences at the GC peak region for analyte and IS using the proposed extraction procedure and chromatographic-MS conditions. The Limits of Detection (LOD) and Quantification (LOQ) were determined experimentally, by analysis of samples spiked with decreasing concentrations of the analytes. LOD was defined as the concentration yielding a signal-to-noise ratio of 3. LOQ was calculated as the smallest concentration of analyte that could be measured with a signal-to-noise ratio of 10.

Recovery

Recoveries of the procaterol was determined for human urine by the analysis of six urine samples spiked at Minimum Required Performance Limit (MRPL ie., 20ng/ml).

RESULTS AND DISCUSSION

Method development and optimisation

Initially, the procaterol is allowed to separate in 5:5 ratio, however, the peak shape of analyte was good. The method was developed according to the WADA ISL and ICH guidelines. The developed method could analyse the Procaterol, which is prohibited in sports under the category of β-2 Agonist. The column allowed separation of analyte in a run time of 23 min. The target compound was identified at R_t of 8.4 min of GC elution with solvent delay of 2 min. Initially, the procaterol is allowed to separate in 5:5 ratio and sample volume of 3 µl, however, the peak shape of analyte was not good. Further, the best separation and acceptable peak shape was achieved at split ratio of 11:1 and sample volume of 2 µl. The column was injected with 2 µl of sample volume through split liner at the split ratio of 11:1 at 280°C avoiding saturation of liner and ensuring optimal vapor-

Table 1: Diagnostic MRM transition for procaterol and ISTD.

Name of analyte	Precursor (m/z)	Product ion	RT	MRM	CE
Procaterol	407	318, 390	8.48	407->318 407->390 407->407	15 20 15
17 a-methyl testosterone (ISTD)	446	301, 195	16.00	446->301 446->195	24 15

Table 2: Results from study of precision and accuracy.

Quality control	Nominal conc. (ng/ml)	Mean± SD (*n*=3)	Accuracy (%)[a]	Precision (%)[b]
Intraday precision				
QC-low	8	8.911±0.083	111%	0.931
QC-medium	15	13.129±0.125	87%	0.952
QC-high	35	33.93±0.256	96%	0.754
Inter day precision				
QC-low	8	8.252±0.141	103%	1.700
QC-medium	15	13.091±0.139	87%	1.060
QC-high	35	33.141±0.355	94%	1.071

[a] Calculated as (mean determined concentration/nominal concentration) x 100
[b] Expressed as % RSD (SD/mean)

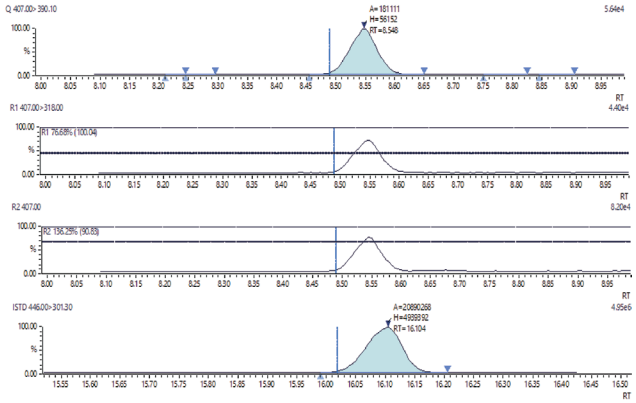

Figure 2: Representative SRM ion chromatograms of prohibited substances in human urine.

ization of the analyte of interest. Detection was performed by positive ion electron impact spray ionization (EI) in Multiple Reaction Monitoring (MRM) mode. The best separation and stability were achieved for the above said conditions. Determination and optimization of the mass spectrometric condition were done in a multistep process. First a full scan spectrum was obtained for every derivatised compound. After selection of a suitable precursor ion, full product scan mass spectra were acquired at different collision energies. Suitable product ion and optimization of the collision energy was then performed both on reference standard and extract from spiked urine samples. The Multiple Reaction Monitoring (MRM) diagnostics ions are shown in Figure 2.

Validation

The calibration curve was constructed using five calibration standards (viz., 5-40 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration and fitted to the y= mx+c using weighing factor (1/X). The regression (r^2) and its equation were found to be 0.98 and y=0.197+0.0002, respectively. Accuracy and precision data for intra- and inter-day samples are ranged from 87-111% and 0.75-1.7%, respectively. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits as given in Table 2. The percentage recovery for procaterol at Minimum Required Performance Limit (MRPL ie., 20ng/ml) was found to be 83%. The recovery was sufficient to reliable identify the procaterol at or below the level prescribed by WADA ie MRPL 20ng/ml. The LOQ and LOD was found to be 5 and 3 ng/ml respectively.

CONCLUSION

A simple and sensitive GC-MS/MS method for quantitative estimation of Procaterol was developed and validated as per the WADA ISL and ICH guidelines. This method was specific, accurate and reproducible for Procaterol. The estimated run time of the developed method is 17 min at constant pressure. The current method would get enhanced by combining the triple quadrupole with Gas Chromatography, thereby can greatly improve the detection capabilities and minimising false detection of target substance in complex matrix. The method would be successfully applied to the screening and confirmation analysis of dope test samples. Further, the extension of this work would be on the differentiation of results of Procaterol analysis in human urine due to presence of confounding/interfering factors. Further, studies are in progress to access the effectiveness of this method for the detection of other β_2-Agonist.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

WADA: World Anti-Doping Agency; **TUE:** Therapeutic Use Exemption certificate; **HPLC:** High Performance Liquid Chromatography; **GC-MS:** Gas

Chromatography- mass spectrometry; **SPE:** Solid Phase Extraction; **LC-MS:** Liquid chromatography mass spectrometry; **TBME:** Tertiary Butyl methyl ether; **R_T:** Retention time.

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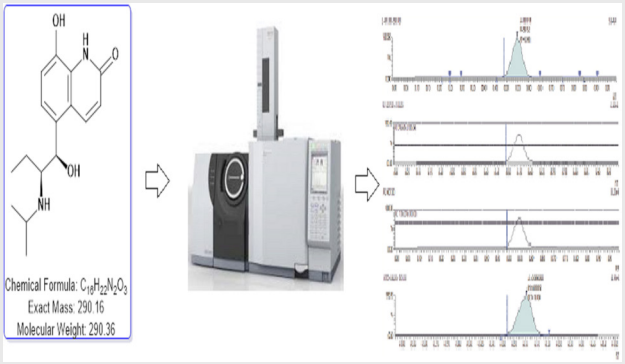
PICTORIAL ABSTRACT



SUMMARY

GC-MS/MS method was developed and validated for Procaterol in human urine as per guideline of ICH and requirements of WADA ISL. This GC-MS/MS method was very satisfactory performance in terms of selectivity, limit of quantification, linearity, recovery, precision and accuracy, in compliance with ICH guidelines. The developed method was simple, precise, accurate and appropriate for the determination of Procaterol in human urine. The method was applied for screening and confirmation analysis of Procaterol in human urine. Furthermore, it would probably be also convenient for the determination of other β_2-Agonist in human urine.

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