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Research Article

LC-MS/MS Estimation of Propranolol level in Exhaled Breath Condensate

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ABSTRACT

Background: Exhaled breath condensate (EBC) could be used as a non-invasive and alternative specimen to urine and blood for monitoring propranolol levels. A simple, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method is employed for the determination of propranolol in EBC samples.
Methods: Samples directly injected to a C_{18} analytical column and isocratically separated using a mobile phase composed of methanol + acetic acid (99:1 v/v). Detection was performed by positive electrospray ionization in multiple reaction monitoring and selected ion recording modes.
Results: The chromatographic separation was obtained within 6.0 min and was linear over the concentration range of 5.6–224.0 ng/mL (R^2 = 0.999). The accuracy and precision of the method were within 15% according to FDA guideline. The found concentrations of propranolol in EBC of two patients receiving 80 mg/day were 30 and 40 ng/mL.
Conclusion: Developed method was applied to determine propranolol levels in three patients receiving propranolol in their medication. The obtained propranolol levels in EBC could be used to develop simpler, cheaper and more feasible analytical methods to be used in routine analysis of propranolol in biomedical analytical laboratories.

Introduction

Propranolol, (2*RS*)-1-[(1-methylethyl) amino]-3-(naphthalen-1-yloxy) propan-2-ol (Figure 1), is a potent β-adrenergic blocking drug belonging to class II of the antiarrhythmics and predominately used as an effective antihypertensive and antianginal agent.[1]

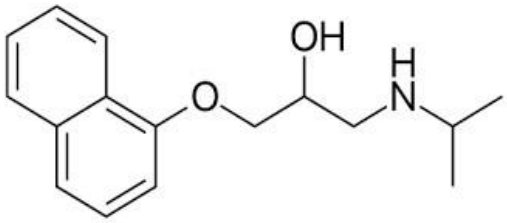

Figure 1. Chemical structure of propranolol.

It has the same affinity for β_1 and β_2 adrenergic receptors, thus, it is a nonselective adrenergic receptor antagonist. Propranolol is a highly lipophilic drug (log P = 2.60)[2] and is almost fully absorbed following oral administration. However, most of the drug is metabolized in the liver during its first pass elimination and averagely 20% of drug reaches the systemic circulation. Propranolol bounds to plasma proteins and distributed in all body tissues.[3] The peak plasma levels of propranolol is observed usually within 3 hr after a 80 mg dosage and fall in the range of 15-180 ng/mL. There is significant inter-subject variations in plasma concentrations after oral administration[4] which require therapeutic drug monitoring (TDM) to avoid adverse effects and obtain the desired clinical benefit. Propranolol toxicity usually results from an

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accidental misuse or overdose of a routine medication. In addition to its medical use, propranolol is sometimes abused by athletes resulting in reducing the heart rate, the contraction force and the coronary flow.[5] Consequently, it is included in the list of prohibited drugs by the world anti-doping agency. Therefore, determination of propranolol in biological fluids is an important issue not only in clinical sciences but also in doping control practice.

Several analytical methods including liquid chromatography (LC), fluorimetry, capillary electrophoresis, gas chromatography (GC) and mass spectrometry were published for propranolol determination in biological samples (Table 1).

The majority of these methods have sample pre-treatment step before introducing the sample into analytical instruments. The important characteristic of an analytical method to be used in clinical and diagnostic is its reliability and rapid determination of analyte in small volumes of biological fluids. Most of the biological samples contain high content of proteins, salts and surfactant like compounds and direct injection of such samples into analytical instruments is not possible. Sample preparation step including the extraction of target analytes from the sample matrix shows an important role in the case of biological samples. Classical sample preparation methods such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) are much time/solvent consuming procedures caused to tough sample work-up.[20,21] Therefore, miniaturization of extraction procedures are required. Dispersive liquid-liquid microextraction (DLLME) is one of the microextraction techniques which has been widely used in bioanalysis and attempt to minimize the use of hazardous solvents is still under consideration.[22] Utilizing a simple biological matrix instead of a complex one saves the time and cost of analysis as over 80% of an analysis process is allocated to sample preparation methodology.

Table 1. Analytical characteristics of the different methods for analysis of propranolol in biological samples.

Method	Extraction procedure; sample volume	Linear range	LOD	Ref.
LC/ fluorometric detection	LLE with 6 mL DCM; plasma/ 1 mL	3.13–100.00 ng/mL	1.56 ng/mL	6
LC–MS/MS	Protein precipitation with 200 µL ACN; plasma/ 100 µL	2-400 ng/mL	ND	7
LC-MS	SPE with Extrelut NT3 column; Biofluids/ 2 mL Tissue/ 200mg	50-5000 µg/L 50-5000 and 1000-50000 ng/g	ND	8
LC/MS	SPE with Oasis® MCX (30 mg; 1 mL) cartridges; whole blood	1–5 µM	0.014 µM	9
LC/UV	LLE with 100 mL isoamyl-alcohol: *n*-heptane [1.5:98.5 v/v]; plasma/ 1 mL	15–180 ng/ mL	10 ng/mL	10
HPLC/ESI-MS	LLE with ethyl acetate; plasma	0.3–200.0 ng/mL	ND	11
HPLC/UV	LLE with 6 mL hexane-*n*-butanol (96:4, v/v); plasma/ 1mL	5.0-100.0 ng/mL	1 ng/mL	12
HPLC-UV	SPE with disposable extraction cartridges; plasma	5-500 ng/mL	1.3 ng/mL	13
HPLC/ESI-MS	SPE with Oasis HLB, (30 mg; 1 mL); plasma/ 300 µL	0.2-135.0 ng/mL	50 pg/mL	14
Spectrofluorimetry	SPE with *N*-vinylacetamide copolymer sorbent; plasma	1.0-75.0 µg/mL	0.046 µg/mL	15
Spectrofluorimetry	Urine/ 100 µL	5-20 µg/mL	0.33 µg/mL	16
GC–MS	SPE with Bond Elut cartridge containing a C_{18}, adsorbent phase (500 mg; 6 mL); plasma/ 3mL	50-300 ng/mL	10 ng/mL	17
GC–MS	SPE; urine	100-2000 ng/mL	ND	18
CE-DAD	Protein precipitation with 1340 µL of ACN and DLLME with 50 µL chloroform and 1.8 mL of the disperser; plasma/ 660 µL	0.02-0.80 µg/mL	0.0041 µg/mL	19

LLE: liquid-liquid extraction; SPE: solid phase extraction; ESI-MS: electrospray ionization mass spectrometry; DCM: dichloromethane; ACN: acetonitrile; DLLME: dispersive liquid- liquid microextraction; ND: no data.

Exhaled breath condensate (EBC) contains the aerosolized droplets and volatile compounds which reflect the airway epithelial lining fluid composition.[23] In human breath, about 3000 volatile substances have been detected. In addition, the EBC sample is known to contain a large number of non-volatile substances coming from the airway-lining fluid.[24-27] Methadone levels in EBC was successfully determined using CE[28] and LC-based[29] analytical methods.

EBC sample collection presents some benefits over the urine and blood samples; *i.e.* ease of operation, no need for skilled operator, less staff time, painless, simple matrix and diminishing the risk of adulteration. Additionally, in a clinical application, repeated samplings within a day is required which is hardly possible for invasive sampling methods. Therefore, EBC sample is a promising biological matrix enabling analyst more convenient in drug monitoring procedure. Using EBC as an alternative biological sample is in its infancy period and only a limited number of drugs were determined in EBC. Knowledge of estimated levels of drugs in EBC may help the pharmaceutical analysts to develop more feasible methods for routine analysis of drugs in EBC samples in the biomedical laboratories.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has confirmed to be an extremely important analytical instrument that provides high sensitive and selective determination with high-resolution chromatographic separation. Urine and plasma samples could not be directly injected to LC–MS/MS system, however there is such a possibility for EBC samples.[30]

The aim of the present work is to provide an accurate and rapid analytical LC-MS/MS method for determination of propranolol in EBC. Direct injection of EBC into LC system avoids prolonged and tedious sample preparation step. This investigation presents further progresses in our efforts on the development an analytical methodology for the direct analysis of drugs in EBC following a simple sampling procedure without any sample pre-treatment and reports propranolol levels in EBC for the first time. By using propranolol levels in EBC, it is possible to develop more simple analytical methods to be used in routine analysis of propranolol in EBC.

Standard solutions & EBC samples

Propranolol powder was purchased from Sobhan Darou Company (Rasht, Iran). Stock standard solution (1000 mg/L) was prepared by dissolving appropriate amount of propranolol in methanol and stored at 4 ˚C. Drug-free EBC samples for method development and validation purposes were provided by volunteers confirmed negative by LC–MS/MS for propranolol. All sample donors signed a consent form approved by the Ethics Committee of Tabriz University of Medical Sciences. The EBC samples were collected by a lab-made cooling trap system[31] and subjects were asked to breathe into cooling system for just five minutes. To avoid the influence of healthy subjects' conditions such as medications and smoking, the subjects did not take any medication and smoking for at least 2 months. Since food and drink may affect the matrix, samples were collected from different persons and pooled. Samples were collected in polypropylene tubes and stored at -20 ˚C until processing. Three EBC samples were collected from patients receiving propranolol and analyzed by developed method (described below).

Separation

Due to the simplicity of EBC matrix, no sample pre-treatment was needed and a 50 µL aliquot was injected directly to the LC–MS/MS system after filtration almost 500 µL of EBC through the 0.2 µm pore size PTFE filter (Chromafil, Germany). The mass spectrometer used in the present work is a Waters Micromass Quattro Micro API 2695. The chromatographic system was an Agilent C_{18} column (150 mm × 4.6 mm, 5 µm particle size) with a mobile phase consisting of methanol + acetic acid (99:1 v/v). The flow rate was 0.50 mL/min. Ionization of analytes was carried out using the electrospray ionization technique with positive polarity (ESI^+) in both selected ion recording (SIR) and multiple reaction monitoring (MRM) modes. SIR is the combination of the particular parent mass and the specific fragment ion and used to selective scanning of the analyte under investigation.

Results and Discussion

The full-scan and SIR MS spectrums for propranolol provided the "fingerprints" used for analyte identification and confirmation. Figure 2A shows the full scan spectrum of spiked propranolol in EBC ($[M+H]^+$ ion at *m/z* 260) under the above described chromatographic conditions. MRM of parent → daughter ion transitions was recorded after collision-induced dissociation fragmentation which was initiated by applying argon gas for collision activated dissociation (best collision energy set of 30.0 eV) to break the precursor ions in order to obtain major product ions. The details of instrumental parameters are shown in Table 2.

Table 2. Instrument settings for the mass spectrometer.*

Analyte	Pecursor ion (*m/z*)	Product ion (*m/z*)	Con voltage (V)	Collision energy (eV)
Propranolol	260	129	35	30

*Source block temperature, 150 °C; desolvation gas temperature, 300 °C; desolvation gas flow, 300 L/hr and capillary voltage, 4.0 kV.

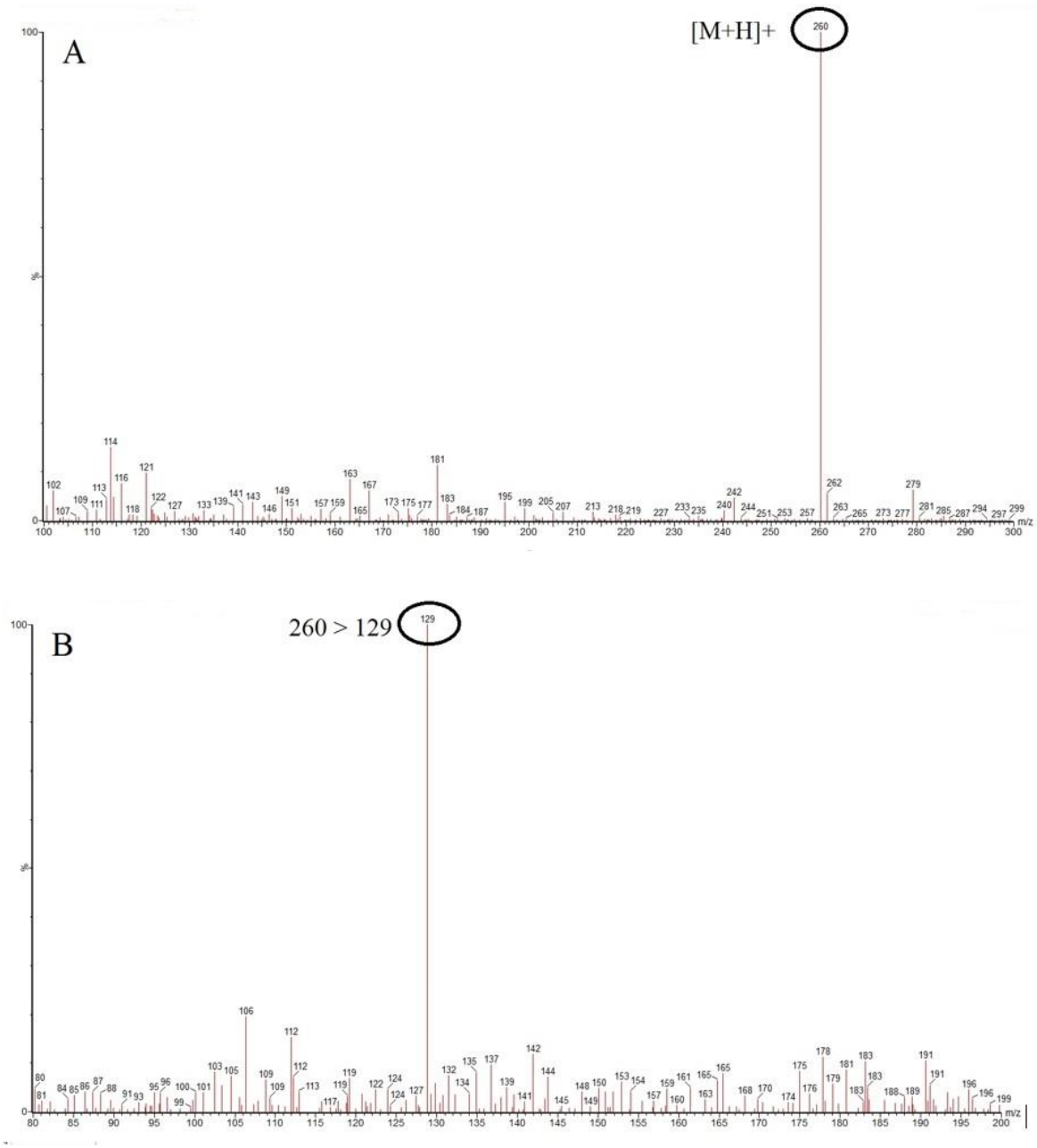

Figure 2. A) Full scan mass spectrum and B) parent to daughter mass spectrum of spiked propranolol in exhaled breath condensate.

The most abundant ion in the obtained mass spectrum at *m/z* 129 (Figure 2B) is ascribed to propranolol daughter ion in MRM mode and quantification was monitored using the MS/MS transition 260 → 129.

Linearity

All quantification steps were performed according to the FDA guideline for bioanalytical method validation.[32] The dynamic range or calibration range of an analytical quantification is defined where the response of the analytical instrument and known levels of sample could be presented by a simple algorithm. This relationship is used for estimation of an unknown sample concentration. As there are no data available on propranolol levels in EBC in clinical practice, the linearity was assessed in the wide range. The six-point calibration curve (mean of three replicates) was constructed by dilution of the stock solution with appropriate volumes. The response of instrument was plotted against the corresponding concentration. The initial and final points in the calibration range are defined as lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), respectively.

The closeness of the results of a set of measurements under the same condition indicates the method precision. The relative standard deviation (RSD)

was used to present the precision. The resulting mathematical relationship from calibration curve was used for calculating the actual concentration of each point in the calibration curve (back-calculated values) in order to estimate the accuracy of the method. The accuracy was simply obtained from the difference between actual and nominal value divided by nominal value for each concentration that should be meet the requirements of the FDA guideline.

Deviations by less than 15% of the RSD is acceptable except for LLOQ where it should be lower than 20%. According to FDA guideline, the mean value should be better than ±15% of the nominal value, except for LLOQ where it could be within ±20%. The mean value of each point in prepared calibration curve was precise and accurate according to FDA requirements. The quantification criteria are listed in Table 3. According to reported equation and its coefficient of determination, the analytical response is proportional to the concentrations of propranolol in the added standard solutions.

Application on real EBC samples

From the experiments, the resulting transition for the quantitative experiment was 260 →129. First scan relies on the $[M+H]^+$ parent ion for propranolol at *m/z* 260 and second scan shows the predominant daughter ion at *m/z* 129 following collision-induced dissociation of the parent ion. Quantification of propranolol was accomplished by monitoring the highest abundance ion (*m/z* 129) in the MS/MS mode.

Propranolol was detected in two of the EBC samples from three patients and chromatogram of EBC sample of patient ≠1 is shown in Figure 3. The amount of propranolol collected from two patient's breath samples was high enough to produce a significant analytical response. The propranolol concentration in EBC of patient ≠3 receiving 40 mg/day of propranolol was lower than acceptable levels for quantification. Table 4 summarizes the results and collected information for the patient EBC samples.

Table 3. Validation data for the proposed LC-MS/MS method for quantification of propranolol in exhaled breath condensate.

Calibration curve equation	R^2 value[a]	Linear range[b]	LLOQ[b]	ULOQ[b]
y =37.15x-9.60	0.999	5.60-224.00	5.60	224.00

[a]Coefficient of determination.
[b]Concentraions are expressed in ng/mL.

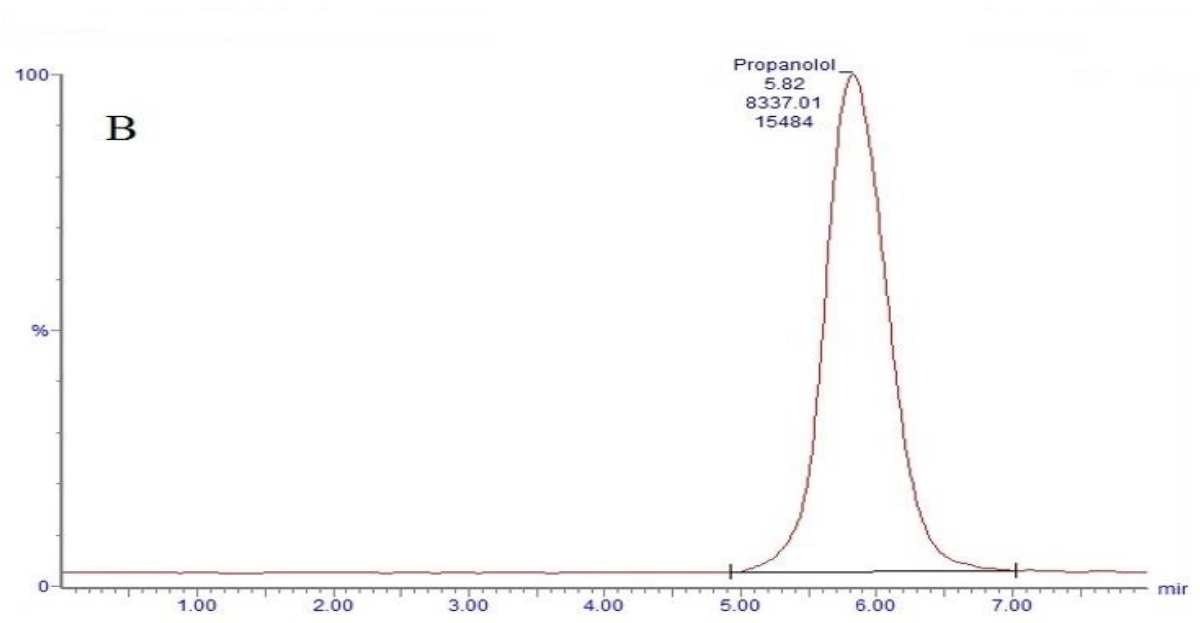

Figure 3. The chromatogram exhaled breath condensate sample was taken from a patient receiving propranolol.

Table 4. Summary of data obtained for propranolol sampled in exhaled breath condensate from three patients.

Case	Propranolol daily dose (mg)	Propranolol concentration in exhaled breath condensate (ng/mL)
1	80	40.72
2	80	30.42
3	40	<LLOQ

Conclusion

This study reports the first observation on propranolol levels in EBC samples collected from patients receiving propranolol. Sampling of EBC is a non-invasive method and normally provides enough specimen volume for analyzing by LC-MS/MS. To minimizing the interference from exogenous/endogenous analytes co-eluted with the intended compound, MS/MS detection was processed. A marked benefit of present method over methods listed in Table 1, is its ability to analyze propranolol in a minimum volume of the biological sample. EBC volume used in our experiment was 500 µL, but just a few microliters are required for submitted into the system. The EBC analysis could be used in TDM or doping tests after full validation of EBC as an alternative samples for blood after conducting the required complementary works.

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Conflict of interests

The authors claim that there is no conflict of interest.

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