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ANALYSIS OF MATABOLITES OF LEVALBUTEROL AND ITS APPLICATION TO *IN VITRO* METABOLISM BY LC-MS

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ABSTRACT

A UPLC-MS method for the quantification of the levalbuterol is described, in addition to its application to the *in vitro* study of metabolism in rat liver microsomes. Protein precipitation extraction was used to extract the sample from microsome samples and the separation was performed on a C18 protected with a guard column of the same type using Water: Acetonitrile with 0.1% Formic acid as the mobile phase, at a flow rate of 0.3mlmin-1. The detection was carried out at 276nm. The method proved to be linear in the range of 2.5-30ngml-1, with a quantification Precision and accuracy, demonstrated by within-day and between-day assays, were lower than 15%. The metabolic study demonstrated that metabolism found two metabolites formed in the incubation mixture of liver microsomes and sample with NADPH, which are identified by LC-MS.

KEYWORDS

In vitro metabolism and LC-MS.

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INTRODUCTION

Methods of measuring drugs in biological media are becoming increasingly important for the study of bioavailability and bioequivalence studies, quantitative evaluation of drugs and their metabolites, new drug development, clinical pharmacokinetics, research in basic biomedical and pharmaceutical sciences and therapeutic drug monitoring. Levalbuterol Figure No.1 (a), chemically 2-(Hydroxymethyl)-4-[(1*S*) 1-hydroxy-

2-(tert-butylamino) ethyl] phenol is a single isomer beta2-agonist that differs from racemic albuterol by elimination of (S)-Albuterol. Levalbuterol leads to activation of beta2-adrenergic receptors on airways smooth muscle leading to the activation of adenylatecyclase, which increases the intracellular concentration of cAMP. The increase in cAMP is associated with the activation of protein kinase A, which in turn, inhibits the phosphorylation of myosin and lowers intracellular ionic calcium concentrations, resulting in muscle relaxation and bronchodilation. Levalbuterol relaxes the smooth muscles of all airways, from the trachea to the terminal bronchioles. Increased cAMP concentrations are also associated with the inhibition of the release of mediators from mast cells in the airways[1-4]. (*R*)-Salbutamol i.e. levalbuterol is metabolised up to 12 times faster than (*S*)-salbutamol.

Literature survey revealed that there is no any UPLC method for levalbuterol[5-9]. For the estimation of the drugs present in the biological fluid, UPLC method is considered to be more suitable since this is a powerful and rugged method. It is also extremely specific, accurate, sensitive and rapid. In this study we have done a metabolite study by using liver microsomes, with a protein precipitation extraction and improved sensitivity for the determination of Levalbuterol in plasma and the developed method is validated as per regulatory requirements.

MATERIAL AND METHODS

Chemicals

Levalbuterol was gifted by FDC Ltd. Mumbai. UPLC grade solvents (Acetonitrile, Methanol) were obtained from Merck and milli-Q water was from SG Series Compact Pretreatment Module.

Instrument

The Waters Acquity UPLC system equipped with a MS detector and an auto sampler was used. Chromatographic separations were performed using the acquity UPLC BEH C-18, 1.7um 2.1 x 100mm column and analyzed by LC software Turbo chrome work station.

Preparation of solutions

50mM ammonium bicarbonate buffer was prepared by dissolving approximately 1.96gm of ammonium bicarbonate in 500ml of water and the pH was adjusted to 7.8 with acetic acid.

Preparation of standard

Levalbuterol and Doxophylline stock solutions were prepared with a concentration of 1mg/ml by dissolving in methanol and the stock solutions were stored in the refrigerator. Spiking solutions of Levalbuterol for the preparation of calibration standards and quality control samples were prepared in methanol and spiked in to the plasma at the ratio of 1:8. The calibration curve was generated using seven calibration standards with the concentrations of 2.5µg/ml (STD 1), 5µg/ml (STD 2), 10µg/ml (STD 3), 15µg/ml (STD 4), 20µg/ml (STD 5), 25µg/ml (STD 6) and 30µg/ml (STD 7). The Quality Control samples were prepared with the concentrations of 5µg/ml (LQC), 15µg/ml (MQC) and 25µg/ml (HQC). The bulk spiked calibration standards and quality control samples were stored in the freezer.

Sample preparation and extraction

Levalbuterol from the plasma was extracted by using protein precipitation extraction technique. Blood samples were collected in heparinised tubes and immediately placed on ice and taken to the lab where they were centrifuged at 5000rpm for 5 min at room temperature. The resulting plasma samples were stored at -75^{o}C until analysis. Aliquot 160µl of plasma into eppindorf tubes and added 20µl of internal standard dilution and vortexed to mix the contents. 20µl of above sample is added and Levalbuterol is extracted by using methanol as a precipitating solvent and vortexed for 30sec. Then the extract was centrifuged at 4^{o}C, 7000rpm for 10 min. The supernatant was taken and transferred to HPLC vials[10].

Preparation of Stock Solution for Metabolite Study

4.551mg drug 10ml (1.5mM)

UPLC method

The mobile phase used was water (phase A) and acetonitrile (Phase B). Before analysis, the mobile

phase was filtered through 0.45µm filter paper and then degassed ultrasonically for 15 min. A gradient method was developed Table No.1. For analysis the mobile phase was initially composed of 90% solvent-A and held for 1 min. The mobile phase composition was then linearly programmed to 90% solvent-B in 20 min and held for 1 min. The mobile phase condition was returned to the starting solvent mixture in 1 min. The analysis was conducted at a flow rate of 0.3 ml/min. The eluent was monitored at a wavelength of 276nm. The total run time was 20 min and injection volume was 5µl. Here Capillary Voltage was 2.97 kV, Cone Voltage was 37 V, Extractor voltage was 2 kV, RF lens voltage was 0.1kV, Source temperature was 120ºC, Desolvation temp was 413ºC, Desolvation flow rate was 700L/hr and Cone flow rate was 25L/hr[11].

In Vitro Metabolism

Sacrifice animals by cervical dislocation, decapitation, or CO_2 asphyxiation.

Remove the liver rapidly and place in ice-cold homogenizing buffer in an ice bath.

Perfuse the liver by inserting a 10-ml syringe and injecting buffer until the effluent is clear and colorless (10 ml buffer for a rat liver).

Remove excess moisture by blotting on paper towels and weigh the tissue.

Add 3 vol of ice-cold homogenizing buffer and mince the liver into small pieces with surgical scissors.

Homogenize, on ice, using a motor-driven Teflon pestle (Potter type) with 10 strokes.

Place homogenate into an ice-cold labeled 1.5-ml centrifuge tube.

Isolation of Microsomes

Centrifuge the homogenate 15 min at 12,500 × *g*, 4°C. The supernatant is the S9 homogenate fraction.

Carefully decant the supernatant into a 1.5-ml ultracentrifuge tube and discard the pellet. Balance pairs of tubes to within 0.01 g using ice-cold homogenizing buffer, pH 7.4

Ultracentrifuge the supernatant 70 min at 105,000 × *g*, 4°C.

Decant the supernatant and resuspend the pellet in 8ml ice-cold pyrophosphate buffer. Homogenize the pellet using the hand-held blender for 3 to 5 sec.

Rebalance the tubes and ultracentrifuge 45 min at 105,000 × *g*, 4°C.

Decant the supernatant, add 6ml ice-cold microsome buffer and resuspend the pellet with a hand-held blender. Further homogenize the pellet with the Potter-type teflon pestle and transfer into a clean tube. Wash the teflon pestle with 2 ml ice-cold microsome buffer and combine.

Determine the protein concentration of a small aliquot by standard methods. Adjust to the desired protein concentration (usually 10 to 20mg/ml) with microsome buffer. Dispense 0.5-ml aliquots into labeled tubes and store at -70° to -80°C.

Methodology for the Determination of Metabolites in Liver Microsomes

Incubation of Levalbuterol with rat liver microsomes was carried out at 37°C in a shaking incubator. The incubation solution contained 0.5ml 100mM potassium phosphate buffer (pH 7.4), 1mg protein/ml microsomes, 2mM NADPH and 1.5mM levalbuterol in a final volume of 100µl. The enzyme reaction was initiated by adding NADPH after an initial 10-min preincubation. The reaction was terminated by adding 1ml of cold methanol. The solution was vortex-mixed and centrifuged at 4°C for 10 min at 3750 rpm. The supernatant was transferred to an epindorff tube and dried under a stream of nitrogen at room temperature. The residue was reconstituted in 200µl of methanol. Metabolites formed are determined by LC/MS[12-14].

***In Vitro* Metabolism of Drug in Rat Liver Microsomes**

1mg protein/ml (Liver Microsomes)

0.5ml 100mM Potassium Phosphate Buffer (pH 7.4)

1.5mM Levalbuterol Solution

2mM NADPH

Incubate in Shaking Incubator for 2Hr

Stop Incubation Reaction with MeOH

Centrifuge, Collect Supernatant and Inject in HPLC and LC-MS.

RESULTS AND DISCUSSION

First metabolite (M_1)

This metabolite obtained after deamination showed a molecular ion at *m/z* 167. The peak eluting yielded a molecular ion at M+1,168.

Second metabolite (M_2)

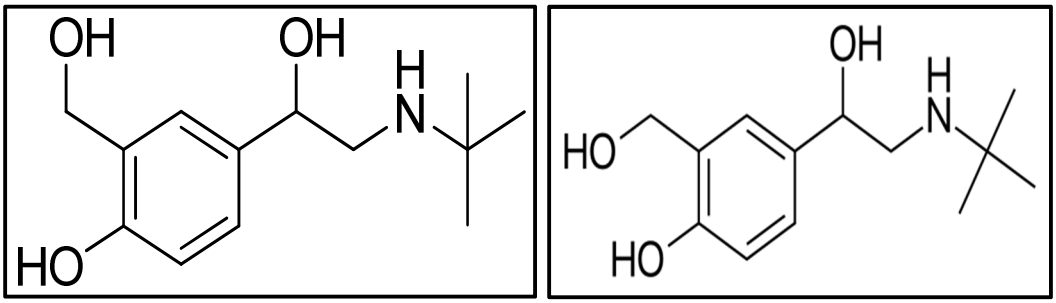
This metabolite peak yielded a protonated molecular ion at *m/z* 221 and ESI mass of this metabolite obtained after dehydration showed a molecular ion at M+1, 222.

Table No.1: LC-MS method for metabolite

S.No	Time (min)	%A	%B
1	0	90	10
2	6	90	10
3	9	40	60
4	16	40	65
5	19	10	90
6	20	10	90

Table No.2: Metabolite

S.No	Name	Levalbuterol	M1	M2
1	RT (min.)	1.1	1.87	3.09



(a) **(b)**

Figure No.1: Chemical structure of (a) Levalbuterol and (b) Albuterol

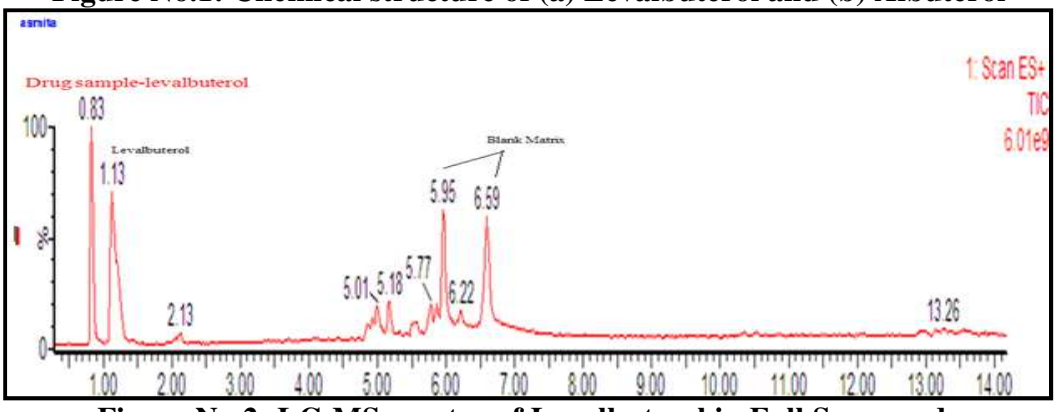


Figure No.2: LC-MS spectra of Levalbuterol in Full Scan mode

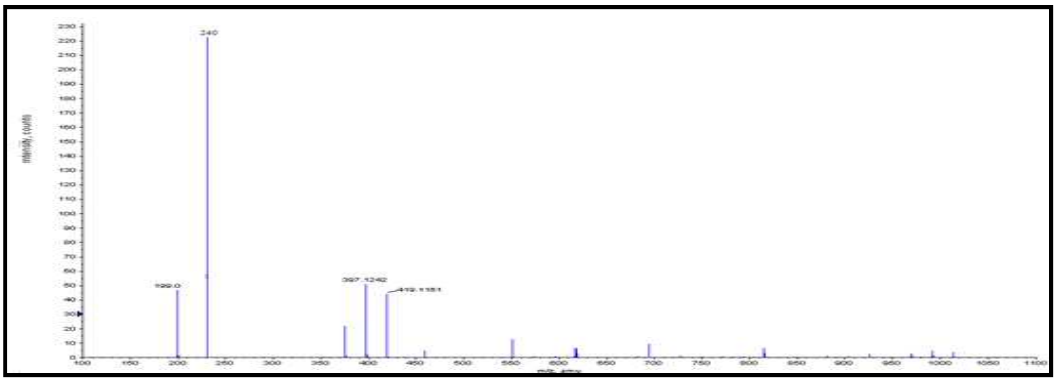


Figure No.3: Mass spectrum of levalbuterol

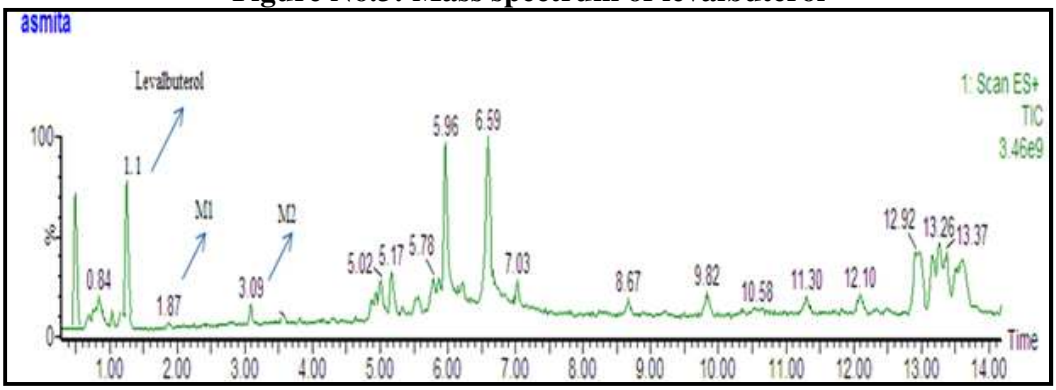


Figure No.4: Representative Chromatogram of metabolites in liver microsomes by LC-MS

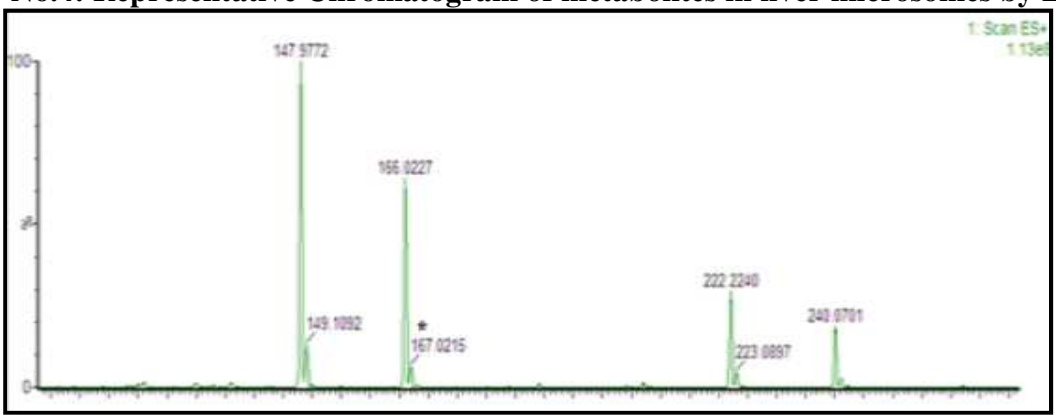
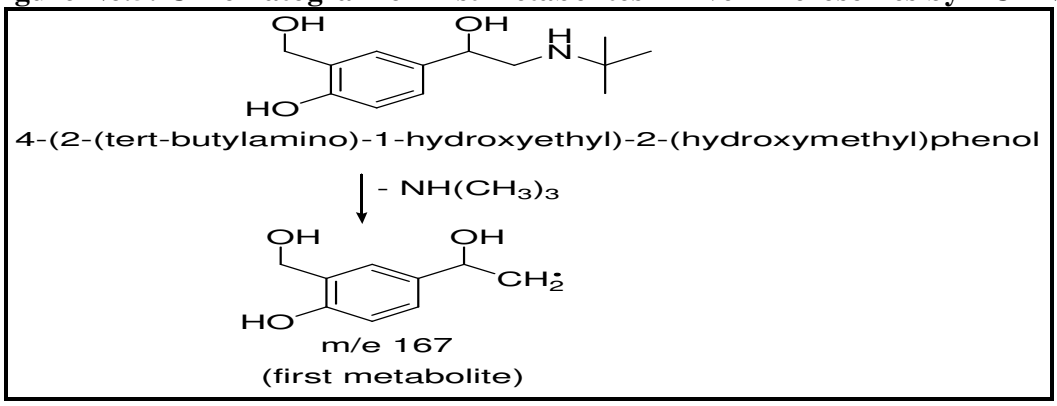


Figure No.5: Chromatogram of first metabolites in liver microsomes by LC-MS



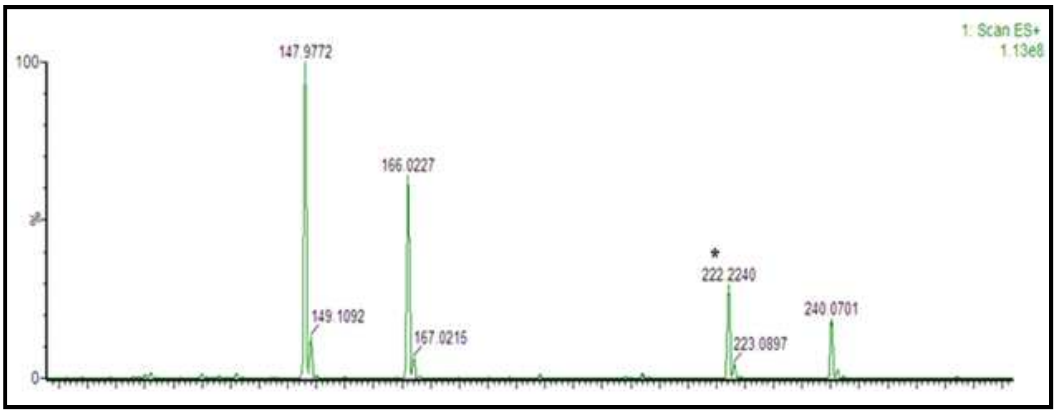
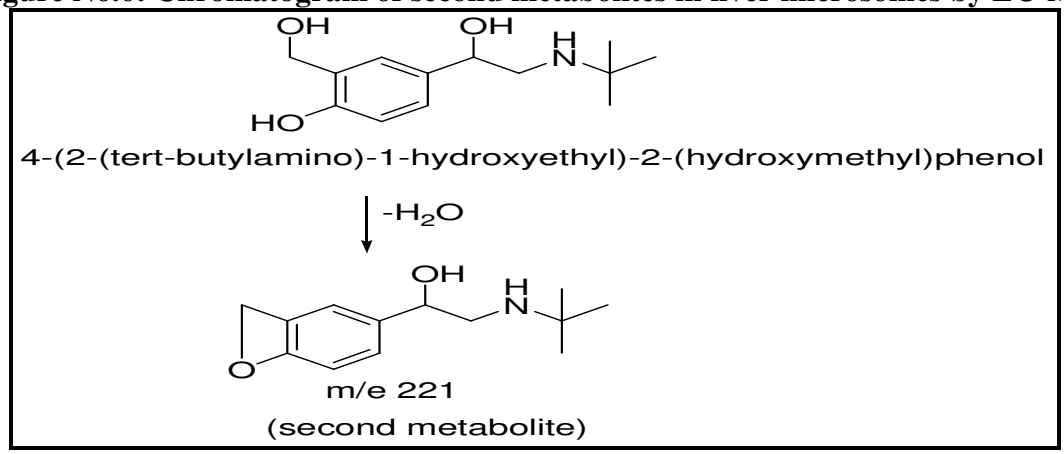


Figure No.6: Chromatogram of second metabolites in liver microsomes by LC-MS



CONCLUSION

The method described here for the quantitation of Levalbuterol in rat plasma is a simple, specific, rapid, accurate and stable LC-MS assay. The method is found to be highly precise and suitable for *in vitro* metabolite study in rat liver microsomes. *In vitro* metabolism study for levalbuterol has been conducted in which two metabolites were detected by HPLC and identified by LC-MS.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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