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# Pharmacokinetic, bioavailability, metabolism and plasma protein binding evaluation of NADPH-oxidase inhibitor apocynin using LC–MS/MS

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ABSTRACT

Apocynin is a major active constituent of *Picrorhiza kurroa* that exhibits potent anti-inflammatory activity by inhibiting superoxide-generating NADPH oxidase enzyme. To elucidate detailed pharmacokinetic profile of apocynin, high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method was developed in rat and human plasma. To the best of our knowledge, this is the first method for complete validation of apocynin in biological matrix using LC–MS/MS. Apocynin was rapidly absorbed after oral administration at 50 mg/kg in rats and peak plasma level achieved within 5 min. Moreover, plasma levels were observed up to 48 h. The bioavailibity of apocynin was found to be 8.3%. In vitro plasma protein binding was found to be 83.41–86.07% and 71.39–73.34% in rat and human plasma, respectively. Apocynin was found stable in gastric (pH 1.2), intestinal (pH 6.8) and physiological (pH 7.4) fluids including microsomal (rat and human) stability studies. Further, apocynin did not convert to its dimeric form diapocynin in any of these studies. The data presented here provide crucial information about apocynin to support its pharmacological efficacy and further development as a potential anti-inflammatory drug candidate.



## 1. Introduction

Apocynin, (Fig. 1a) also known as acetovanillone (4-hydroxy-3-methoxy-acetophenone), is the major active constituent of ayurvedic and Chinese medicinal herb *Picrorhiza kurroa* [1]. Traditionally *P. kurroa* has been used to treat hepatic diseases, respiratory tract disorders, diarrhea, epilepsy and fever. Apocynin is a specific inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and exhibits potent anti-inflammatory activity [2,3]. Apocynin is also useful as neuroprotectant and plays a crucial role in pathogenesis of brain injury and neurological dysfunction [4].

Despite several pharmacodynamic properties, very limited work has been reported on pharmacokinetics of apocynin. Pharmacokinetics and metabolism studies play a foremost role in drug discovery and development process [5–8]. There were few publications in the literature regarding apocynin pharmacokinetic data. For instance, Ximenes et al. reported that activity of apocynin was due to its active metabolite diapocynin (Fig. 1b) through peroxidases in in vitro assay [9]. But Wang et al. reported that apocynin is metabolized to glycoconjugate but not to diapocynin based on single ion monitoring. However, single ion monitoring (LC–MS) makes the studies less selective and specific compared to latest multiple reaction monitoring techniques. It remains unclear that NADPH oxidase inhibition is due to apocynin or diapocynin or both [10]. Wang et al. reported a HPLC–UV method for the pharmacokinetic evaluation of apocynin but the method had a drawback of low sensitivity (LLOQ – 200 ng/mL) and selectivity, resulting in the estimation of apocynin plasma concentration for only 20 min post-oral administration [11]. The plasma concentration time profile generated by HPLC–UV method was insufficient for reliable estimation

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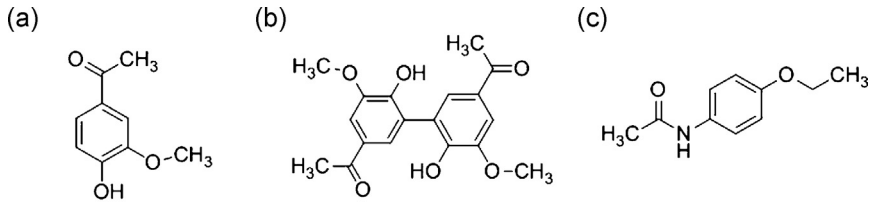


**Fig. 1.** Chemical structures of (a) apocynin, (b) diapocynin, and (c) phenacetin (IS).

of the low concentrations at terminal elimination phase and pharmacokinetic parameters. Earlier reported methods were limited by their inability to characterize the in vivo and in vitro behavior of the apocynin. To investigate its pharmacokinetics and evaluate whether diapocynin is a metabolite of apocynin or not, there is a need to develop a better and advanced bioanalytical method for the analysis of apocynin in the biological fluids.

Thus, the aim of present study was to develope a sensitive and selective liquid chromatography mass spectrometry (LC–MS/MS) method for quantification of apocynin and to account its ADME properties along with detection of its metabolite, diapocynin. In this study we report pH dependent stability, metabolism and plasma protein binding of apocynin along with in vivo pharamacokinetic data in *SD* rats. These findings in correlation with biological response would be useful for developing apocynin as a safe and efficacious anti-inflammatory agent.

## 2. Experimental

### 2.1. Chemicals and reagents

Apocynin (purity ≥ 98%), phenacetin, testosterone, verapamil, magnesium chloride hexahydrate, sodium chloride and DSC-18 cartridges (Discovery Supelco Cat. no. 52602-U) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Diapocynin (HPLC purity ≥ 98%) was synthesized by author from Medicinal and Process Chemistry Division, CSIR-Central Drug Research Institute, Lucknow. HPLC grade acetonitrile was purchased from Merck Specialities Pvt Ltd., Mumbai, India. Sodium heparin injection IP (25,000 IU in 5 mL vial) was procured from the Gland Pharma Ltd., Hyderabad, India. Potassium chloride, potassium dihydrogen phosphate, and nicotinamide adenine dinucleotide phosphate reduced, tetrasodium salt (NADPH), glycerol, sodium hydroxide were purchased from SRL Pvt Ltd., Mumbai, India. Ultrapure water of ≥18 mΩ cm was obtained from a Milli-Q PLUS PF water purification system. Human microsomes (pool of 50 donors) were purchased from In-Vitrogen, USA. Drug-free rat plasma was collected from adult healthy male *Sprague-Dawley* (*SD*) rats procured from National Laboratory Animal Center, CSIR-CDRI, Lucknow, India. All the animal experiments were conducted in accordance with current legislation on animal experiments as per Institutional Animal Ethics Committee at CSIR-Central Drug Research Institute (IAEC approval no IAEC/2012/91Ns). Human plasma was purchased from Bioreclamation, USA. All other reagents were of analytical grade and purchased from standard chemical suppliers.

### 2.2. Diapocynin synthesis

Synthesis of diapocynin from apocynin is an oxidation–reduction reaction, requiring the in situ generation of sulfate radicals, which remove one hydrogen from each molecule of apocynin, producing diapocynin. A modified protocol was used for the synthesis of diapocynin from apocynin [10,12,13].

### 2.3. Stock, standard and quality control sample preparation

Stock solutions (1 mg/mL) of apocynin, diapocynin, phenacetin, testosterone and verapamil were prepared in acetonitrile. The calibration standards of apocynin and diapocynin were prepared by step-wise dilution of the stock solution in acetonitrile with large dynamic range of 1–1000 ng/mL. Quality control samples (QCs) at four different concentrations used as, lower limit of quantification (LLOQ – 1 ng/mL), low quality control (LQC – 5 ng/mL), middle quality control (MQC – 480 ng/mL) and high quality control (HQC – 750 ng/mL), were prepared separately in five replicates, independent of the calibration standards. Phenacetin (internal standard, IS) was diluted to 500 ng/mL in acetonitrile for spiking into plasma. Calibration curve (CC) and QCs samples were prepared by spiking working stocks (less than 5% of the total plasma volume) in blank plasma to establish the final CC range of 1–1000 ng/mL. All the stocks were found stable for complete validation and other studies.

### 2.4. Sample processing

Rat plasma samples (pharmacokinetic, plasma protein binding (PPB) and validation studies) and human plasma samples (validation and PPB studies) were processed using solid phase extraction (SPE) technique. The CC and QCs were prepared by spiking 5 μL of appropriate calibration stock in 95 μL of blank plasma. To the calibration standards, QCs, pharmacokinetic samples and receiver buffer samples from the PPB, 20 μL of IS solution (final concentration 500 ng/mL) was added. All these samples were further diluted with 500 μL of 10 mM ammonium acetate buffer (AAB), pH 6.0 and vertex mixed for 5 min. Prior to the extraction, the cartridges were pre-conditioned with 2 mL of acetonitrile and 2 mL of AAB followed by diluted samples loading into the SPE cartridges. After loading, cartridges were washed with 1 mL of AAB to remove endogenous plasma matrix. Analyte elution was carried out with 2 mL of acetonitrile in fresh glass tubes and dried under a gentle stream of nitrogen at 50 °C. The dry residues were finally reconstituted with acetonitrile (100 μL) and supernatant (10 μL) was injected into LC–MS/MS analysis.

### 2.5. LC–MS/MS method development

Method development, validation and sample analysis was performed on an API-4000-Q-Trap Mass Spectrometer, Applied Biosystems (Foster City, CA) equipped with Prominence HPLC from Shimadzu Co. (Columbia, MD). A Thermo Hypersil $C_{18}$ column (Betasil, 50 mm × 2.1 mm, 3 μm) was used for separation of apocynin, diapocynin and phenacetin (IS). The system was calibrated in isocratic mode with a mobile phase consisting of acetonitrile: 10 mM ammonium acetate buffer (pH 4.0) (80:20, v/v) at a flow rate of 0.3 mL/min. The mass spectrometric detection was performed in the multiple reaction monitoring mode (MRM) using an electro-spray ionization (ESI) source in negative mode with gas 1, gas 2, and curtain gas set at 60, 50, and 15 psi, respectively. The source temperature and ion spray voltage were