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Validated and optimized RP-HPLC method for the simultaneous quantification of meloxicam and its major metabolites in biological fluids with liquid liquid and solid phase extraction technique

Frist Name	Middle Name	Last Name	Academic Degree	Affiliation	Email Address
Yasar		Shah	Ph.D	Department of Pharmacy, University of Swabi, Swabi	shah.yasar@gmail.com
Ibrahim		Khadra	Ph.D	Institute of Pharmacy and Biomedical Sciences, Uni	Ibrahim.khadra@strath.ac.uk
Zia		Ullah	Ph.D	Department of Pharmacy, University of Peshawar, P	razmian_1433@yahoo.com
Naila		Shahbaz	Ph.D	Department of Pharmacy, University of Peshawar, P	nailashahbaz55@yahoo.com
Fazle		Khuda	Ph.D	Department of Pharmacy, University of Peshawar, P	fazlikhuda2012@upesh.edu.pk
Zafar		Iqbal	Ph.D	Department of Pharmacy, University of Peshawar, P	zafar_iqbal@upesh.edu.pk
Aman		Ullah	Ph.D	University of Peshawar	amankhanphd@gmail.com
Lateef		Ahmad	Ph.D	Department of Pharmacy, University of Swabi, Swabi	lateefswabian@gmail.com
Ismail			Ph.D	Department of Pharmacy, University of Peshawar, P	ismailzaidawal@gmail.com
Muhammad		Hassan	Ph.D	Department of Pharmacy, University of Peshawar, P	hassanmohammad63@hotmail.com

Abtract

Meloxicam is the most commonly prescribed non-steroidal anti-inflammatory drug. In this study, a simple, rapid and cost-effective method for the analysis of meloxicam and its major metabolites (5-hydroxy meloxicam and 5-carboxy meloxicam) in biological fluids (human plasma, urine and saliva) was developed and validated using RP-HPLC coupled with UV detector. The samples were analyzed by injecting 20 µl into the HPLC system using supelco analytical C18 (150 mm ×4.6 mm, 5 µm) column, protected by a C18 (30 mm×4.6 mm, 10 µm) Perkin Elmer, guard column. The mobile phase methanol: TFA (0.05% aqueous solution) in 60:40%v/v was pumped with a flow rate of 1.3 mL/min at ambient temperature and the eluents were checked at 353nm using Piroxicam as internal standard. Meloxicam and the metabolites were extracted from biological fluids using dichloromethane and the percent recovery for meloxicam, 5-hydroxy meloxicam and 5-carboxy meloxicam were 98.8%, 97.3%, 97% in plasma, 99%, 98.6%, 95.1% in urine and 95.8%, 92.9%, 92.7% in saliva, respectively. The limits of detection of meloxicam, 5-

hydroxy meloxicam and 5-carboxy meloxicam were 3 ng, 10 ng and 8 ng, whereas limit of quantification were 9 ng, 30 ng and 25 ng, respectively. The method was linear over the concentration range of 10 - 2000 ng/mL for meloxicam, 30 - 1000 ng/mL, 25 - 1000 ng/mL for 5-hydroxy meloxicam and 5-carboxy meloxicam, respectively. The developed method was validated according to standard guidelines, various experimental parameters and chromatographic conditions such as mobile phase composition, flow rate, linearity, accuracy, precision, sensitivity etc. were optimized and were successfully applied for the pharmacokinetic studies in the plasma samples of the healthy human volunteers.

1. Introduction

Meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-*2H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide] a yellow crystalline powder, with a molecular weight of 351.40 g/mole and is insoluble in water, with a slightly solubility in organic solvents i.e. methanol and ethanol [1, 2]. Chemical structure of meloxicam and its major metabolites are presented in Fig-1.

Meloxicam is an NSAID from oxicam group, it preferentially inhibit cyclooxygenase-2 (COX-2) with comparative to cyclooxygenase-1(COX-1) [3]. Unlike other NSAIDs, meloxicam is gentle on stomach with low ulcer-genic property, due to lower affinity for COX-1 [4]. It is mainly used in the management of ankylosing spondylitis, arthritis, rheumatoid arthritis, and other rheumatologic conditions [5].The bioavailability of meloxicam is 89% and 99% of the absorbed drug bind to the plasma protein, the peak plasma concentration is achieved in 5 – 6 hours and elimination half-life is about 20 hours [6-9].It is metabolized by the hepatic CYP2C9 and to a lesser extent by CYP3A4 to 5-hydroxy meloxicam which is further converted to 5-carboxy meloxicam [9, 10] that are rapidly excreted in urine [8]. Whole of administered meloxicam is excreted as metabolites in urine and in feces and only minimal amounts of parent drug can be detected in urine and in feces [11].

Several methodical approaches have been reported for the analysis of meloxicam in the biological samples, these techniques include HPLC and LCMS**.** Reported methods applied advanced detectors like diode array detector and mass spectrometry [12 -14], which are not readily and easily available. Moreover, mobile phase composition used in all of the methods is complex, consisting of buffers which can affect the column life and processing time [12, 15, 16]. Extraction of meloxicam from biological samples is a tedious job and most of the methods have used large

volumes of plasma without any proper extraction procedure [4, 13]. Not a single method of HPLC for the simultaneous analysis of meloxicam and its metabolites (5-hydroxy meloxicam and 5-carboxy meloxicam) has been reported in biological fluids. There is a need to develop a validated, sensitive, easy and rapid method for the estimation of meloxicam and its metabolites in biological samples, particularly for the pharmacokinetic studies.

In the presented study, suitable extraction procedure has been developed for extraction of meloxicam and its metabolites from biological samples. The developed method is a novel one with respect to mobile phase composition and shorter analysis time. The suggested method was then successfully applied for the routine analysis of meloxicam and its metabolites in pharmacokinetics study of the drug.

2. Experimental

2.1. Chemicals

Meloxicam was provided by Sigma Aldrich, 5-carboxy meloxicam and 5-hydroxy meloxicam was purchased from Toronto Research Chemicals (Canada), Piroxicam was a kind gift from Medicraft Pharmaceuticals (Peshawar, Pakistan). The analytical grade HPLC solvents Methanol, Trifluroacetic-acid (TFA) and dichloromethane were procured from Sigma Aldrich (Oslo, Norway). Freshly, double distilled and deionized HPLC grade water was obtained from Millipore distillation apparatus (Milford, USA).

2.2. Equipment's

The analysis were performed using HPLC system (Perkin Elmer Series 200, Norwalk, USA) consisting of a pump, column oven, UV detector, a vacuum degasser, a rheodyne manual injector with 20 μL loop which is linked to a Pe Nelson NCI-900. The chromatographic setup was run by a Total Chrome Workstation (version 6.3.1) Perkin Elmer software for the solicitation and proceeding of data. The column used for analysis of the drug was Supelco C18 reverse phase HPLC column (150mm x 4.6mm ,5μm) and guarded by RP Perkin Elmer C18 (30mm × 4.6mm, 10μm; Norwalk, USA) column. Supel clean SPE cartridges LC-18 (1ml; particle size 56.2μm; Bellefonte, USA) with the Visiprep^{TM} manifold vacuum were used for solid-phase extraction. A Schimadzu (AX 200) electronic balance, temperature controlled centrifuge machine (model: k-2080, Centurion, UK) was used.

2.3. Chromatographic conditions

The mobile phase was comprised of methanol: 0.05% TFA (60:40% v/v), pumped at the flow rate of 1.3ml/min. These solvents were filtered through membrane (0.2µm) and degassed by ultra-sonication. Piroxicam was used as internal standard and the eluents were monitored at 353 nm.

2.4. Preparation of standard solutions

The standard stock solution of meloxicam (0.5mg/ml) were prepared in methanol containing 20µl of glacial acetic acid. The standard stock solution of 5-hydroxy meloxicam, 5-carboxy meloxicam and piroxicam (0.5mg/ml) were also prepared in methanol, DMF (dimethyl-form-amide) and methanol, respectively and further dilutions were prepared using mobile phase.

The working solutions of meloxicam (10, 50, 100, 250, 500, 1000, 1500, 2000ng/ml) were prepared by diluting the standard secondary stock solution of meloxicam (10µg/ml). The working standard solutions of 5-carboxy meloxicam (25, 50, 75, 100, 250, 500, 750, 1000 ng/ml), 5-hydroxy meloxicam (30, 50, 75, 100, 250, 500, 750, 1000ng/ml) and that of piroxicam (1000ng/ml) were prepared from their respective standard secondary stock solution (10µg/ml) and were stored at refrigerator temperature.

2.5. Sample collection and preparation

The study was approved by the Ethical Committee of the Department of Pharmacy, University of Peshawar and informed written assent was attained from the recruited healthy human volunteers, aged between 25 to 35 years and BMI in the range of 23-30. The volunteers were instructed to avoid any medicine for two weeks prior to the study. Early morning at fasting condition, a single oral dose of the meloxicam was taken orally with a glass of water ($\approx$ 250 ml) and blood (2ml), urine (20 ml), saliva samples (3 ml) were collected periodically, centrifuged at 3000 rpm for 10 min; transferred into eppendorf tubes and stored at -20 °C till analysis.

The blank biological fluids (collected before administration of the drug) were thawed at room temperature, at the time of analysis and spiked with appropriate concentration of working solution of meloxicam (i.e., 10, 50, 100, 250, 500, 1000, 1500, 2000 ng/ml), 5-hydroxy meloxicam (i.e., 30, 50, 75, 100, 250, 500, 750, 1000ng/ml), 5-carboxy meloxicam (i.e., 25, 50, 75, 100, 250, 500, 750, 1000 ng/ml) and piroxicam (internal standard) to obtain calibration standards.

2.6. Extraction of meloxicam and its metabolites from biological samples

Liquid-liquid and solid-phase extraction were assessed for the recovery of parent drug and its metabolites from biological samples.

2.6.1. Liquid-liquid extraction

Biological fluids (200µl) were mixed with internal standard (100µl = 100ng) in eppendorf tube, acetonitrile (600µl) was added and vortexed for 3min. Then dichloromethane (1100µl) was added, further vortexed for 5min, centrifuged for 15min at 9000 rpm, organic layer collected and was evaporated under the gentle stream of nitrogen. The residues were re-dissolved in methanol (2ml) and injected into HPLC for analysis.

2.6.2. Solid phase extraction (SPE)

SPE procedure was also utilized for the biological samples in order to achieve good recovery of analytes and comparison between the two methods. In SPE, first conditioning of the cartridge was carried out using methanol (1 ml) followed by equilibration with 1ml of water. The biological sample (200 µl) containing internal standard (100 µl), water (3 ml), concentrated HCl (10 µl) were transferred into the cartridge and forced through it by applying vacuum, washed with water (2 ml), dried by air and then dichloromethane (1 ml) was used to elute the analytes. The

eluate was then dried under the stream of nitrogen gas, final volume was made up to 2 ml with methanol and 20 μl was introduced into the HPLC system.

2.7. Chromatographic conditions optimizations

Different chromatographic parameters like mobile phase composition ratio, flow rate, wavelengths of detector, injection volumes, internal standard and extraction solvents were optimized for quantitative determination of the meloxicam and its metabolites, using RP-HPLC method in an isocratic mode.

2.7.1. Mobile phase composition

A number of organic solvents such as acetonitrile, methanol, trifluoroacetic-acid (TFA), phosphate buffer, pH adjusted distilled water were tried in different combinations both in gradient and in an isocratic mode for the determination of the meloxicam and its metabolites. The mobile phase composition which demonstrated good resolution and separation was selected for analysis.

2.7.2. Flow rate composition

The flow rate significantly affect the sample elution, resolution, separation and peak shape. In this method flow rates in different ranges i.e. 0.8-1.5ml/min were evaluated and one that gave best resolution was selected as optimum flow rate.

2.7.3. Detection wavelength

A Perkin Elmer UV lambda (Nowak, USA) was used to determine the maximum wavelength of maximum absorbance (λ_{max}) of meloxicam. Effect of wave length on analytes response was studied by varying wave length in the range of 330 – 355 nm.

2.7.4. Injection Volumes

Different injection volumes in the ranges of 10 µl-50 µl were assessed using rheodyne manual injector.

2.7.5. Internal standard selection

Different internal standards such as naproxen sodium, lumefantrine, diclofenac-sodium, and piroxicam were tried. In present studies the Piroxicam showed maximum response and better chromatographic properties, therefore it was selected as internal standard.

2.7.6. Selection of extraction solvent

Various solvents such as diethyl-ether, dichloromethane, acetonitrile and n-hexane were evaluated as extraction solvents. Optimum percent recovery was achieved with dichloromethane as an extraction solvent.

2.8. Validation of the method

Validation of the developed method was carried out according to ICH guidelines with special emphasis on specificity, linearity, precision, accuracy, robustness, selectivity, limit of detection (LOD), and limit of quantification (LOQ) [17].

2.8.1. Specificity

The specificity of developed method was judged from the complete peak resolution of the meloxicam and its metabolites in mobile phase, spiked biological sample in a 1:1 mixture (i.e. 1 µg/ml of the sample as well as internal standard).

2.8.2. Linearity

The linearity was evaluated by scheming calibration curves, at eight concentration level of meloxicam (10, 50, 100, 250, 500, 1000, 1500 and 2000 ng/ml), 5-hydroxy meloxicam (30, 50, 75, 100, 250, 500, 750 and 1000 ng/ml), 5-carboxy meloxicam (25, 50, 75, 100, 250, 500, 750 and 1000 ng/ml) and piroxicam (1000ng/ml). Following analysis, the ratios of peak area of the analyte and internal standard was plotted as function of the concentration of analyte using the squares linear least regression equation and the correlation co-efficient.

2.8.3. Accuracy

The accuracy of the developed method was calculated from the percent recovery of the spiked sample. Blank human biological sample was spiked with analytes of different concentration levels keeping the concentration of I.S. constant. The similar concentrations levels were also prepared in mobile phase. Samples were then injected in triplicate and percent recoveries were calculated by the following equation:

$$\text{Percent recovery} = \frac{A}{B} \times 100$$

Where

A = Response ratio of the peak of spiked biological sample (analyte/I.S)

B = Response ratio of the peak of sample in standard solution (analyte/I.S)

2.8.4. Precision

Precision of the developed method was determined by injection and the analysis repeatability. The injection precision repeatability was determined by injecting biological sample, spiked with 1000ng/ml of meloxicam, 5-carboxy meloxicam and 5-hydroxy meloxicam for 10 times into HPLC. The peak area and the retention time was obtained and articulated as mean, standard deviation (±SD), and covariance (% RSD). The precise analysis repeatability of the method was analyzed by spiking six biological samples with 1000 ng/ml of meloxicam, 5-carboxy meloxicam and 5-hydroxy meloxicam, independently.

Intermediate precision (i.e. intra-day and the inter-day studies) was determined by injecting plasma sample spiked with appropriate standard concentration of the analyte and internal standard on 8.0, 16.0 and 24.0 hours for three successive days. The outcome was depicted as mean, standard deviation as well as covariance and the following equation was employed to determine the concentration

$$C = \frac{X}{Y} \times \frac{A}{B} \times C_S \times D_F$$

Where

x = Peak area of the analyte in biological sample

y = Peak areas of the I.S in biological sample

A = Peak area of the I.S in 1:1 mixture

B = Peak area of the analyte in 1:1 mixture

Cs = Concentration of analyte in the 1:1 mixture

D_F = is the dilution factor

2.8.5. Sensitivity

The developed method sensitivity was assessed in terms of LOD and LOQ of analytes. The LOD was determined from the peak of the analyte by the software at which signal to noise ratio is 3 and the LOQ was concentration of the analyte at which signal to noise ratio is equal to 10.

2.8.6. Stability

Stability studies of the meloxicam and its metabolites were assessed in spiked biological samples and standard stock solution at room temperature (25°C), refrigerator temperature (2-8°C) and at -20°C for one month. Each sample was then analyzed (n = 3) and the following equation was used to evaluate the stability:

$$\text{Percent Stability} = \frac{St}{So} \times 100$$

Where

St =Sample stability at that time

So=Initial stability of sample

2.8.7. Robustness/ Ruggedness

The developed method robustness/ruggedness was investigated by bringing deliberately small alterations in chromatographic conditions, such as composition of the mobile phase ($\pm$ 2%), temperature of the column oven ($\pm$ 5°C), mobile phase flow rate ($\pm$ 0.2 ml/min) and detection wavelength ($\pm$ 5%).

3. Results and discussion

The developed method for the analysis of meloxicam and its metabolites in human biological fluids using piroxicam as I.S is novel, fast, economical and sensitive. All the analytes and internal standard were completely resolved. Different chromatographic conditions and various experimental parameters of the developed method were optimized and validated according to ICH guidelines. According to literature it is first attempt to analyze the meloxicam and its metabolites simultaneously in biological samples.

3.1 Sample preparation

Meloxicam is sparingly soluble in methanol, ethanol, and however it was better in methanol that was further improved by the addition of 20µl of glacial acetic acid to about 10 ml of solution. The stock solution was then diluted with methanol.

The protein in the plasma samples were precipitated with acetonitrile using three times of the plasma volume. The extraction of the analytes from the biological samples were carried out with dichloromethane, chloroform, ethyl acetate, diethyl ether, n-hexane either alone or with different combinations. Dichloromethane showed better percent recovery in both extraction procedure and was selected for extraction of analytes from the biological samples.

3.2. Chromatographic conditions of the method

3.2.1. Mobile phase and composition

Different solvents such as acetonitrile, methanol, TFA, water and pH adjusted water were evaluated as mobile phases in different compositions. First acetonitrile and water (50: 50%v/v) as mobile phase was used, showed good shape and resolution of the peaks but retention time was not stable. The water was replaced with pH adjusted water in the range of 2 – 4 and it ends with the same results of stability of retention time. Various ratio of the methanol: 0.05%TFA was evaluated as the mobile phase to achieve better separation and stable chromatogram of the analytes. The results showed better resolution, peak shape and stability of the methods when methanol and TFA (0.05%) was used in 60:40% v/v.

3.2.2. Flow rate

The mobile flow rates ranges from 0.8-1.5% were applied; the optimum flow rate 1.3 ml/min was selected that gave good resolution of the analytes and total elution time was less than 7 min.

3.2.3. Selection of the wavelength

Wavelength 330 nm, 342 nm, 353 nm and 355 nm were evaluated and the best response for meloxicam, its metabolites and internal standard was observed at 353 nm.

3.2.4. Selection of the internal standard

Various compounds were assessed as internal standard that include naproxen sodium, diclofenac sodium, lumefantrine and piroxicam. Among these piroxicam showed good resolution, sensitivity, specificity, recovery, stability, and while other compounds were having long retention time or poor recovery.

3.2.5. Selection of the column

Thermo Quest Hypersil C18 column (250×4.6mm, 5µm; Run-corn, UK), Brownlee Perkin Elmer analytical C18 (150×4.6mm, 5µm; Shelton, USA) column, Supelco/discovery C18 column (150x4.6mm, 5µm), Supelco/discovery

C18 (250x4.6mm, 5µm) column and Thermo Quest Hypersil C8 (150×4.6mm, 5µm; Run-corn, UK) column were tried. The best resolution and other chromatographic properties were obtained with supelco discovery analytical C18 column (150 x 4.6mm, 5µm). The representative chromatogram of the standard sample spiked with meloxicam, 5-hydroxy, 5-carboxy meloxicam and I.S on the supelco/discovery C18 column is shown in Fig. 2.

3.3. Method validation

3.3.1. Linearity

The calibration curves of meloxicam(10-2000 ng/ml), 5-hydroxy meloxicam (30-1000 ng/ml) and 5-carboxy meloxicam (25-1000 ng/ml) in spiked biological samples demonstrated excellent linearity (r^2 = 0.999) results are depicted in Table 1 and in Fig. 3.

3.3.2. Selectivity

The selectivity of the method for the meloxicam and its metabolites was determined from a) mobile phase, b) blank plasma, urine and saliva c) plasma, urine, saliva sample spiked with meloxicam, 5-hydroxy meloxicam, 5-carboxy meloxicam and piroxicam as I.S, d) plasma, urine, saliva sample spiked with I.S. The Fig. 4, 5, 6 confirms the good resolution of the peaks and no other exogenous or endogenous peaks interfere with the chromatograms.

3.3.3. Precision

The precision results obtained from the injection repeatability and the intermediate precision (inter-day, intra-day studies) are summarized in Table 1 and 2. The method demonstrated good precision, the % RSD value for both inter-day and intra-day which was less than 2%.

3.3.4 Accuracy

The developed method accuracy was assessed from the percent recovery, the mean percent recovery of meloxicam, 5-hydroxy meloxicam, 5-carboxy meloxicam were 98.8%, 97.3%, 97% in plasma 99%, 98.6%, 95.1% in urine and 95.8%, 92.9%, 92.7% in saliva by using liquid-liquid extraction and 92%, 84%, 77.8% in plasma, 93.4%, 87%, 83.1% in urine, 85.5%, 78%, 74% in saliva by using solid-phase extraction showing %RSD value less than 1%, represents that the developed method was accurate. Percent recovery of all the analytes was higher with liquid-liquid extraction than SPE.

3.3.5 Sensitivity

The LOD for meloxicam, 5-hydroxy meloxicam and 5-carboxy meloxicam were 3ng, 10ng and 8ng, respectively. The LOQ were 9ng, 30ng and 25ng, respectively. Chromatograms of LLODs and LLOQs are shown in Fig. 7 and Fig. 8.

3.3.6 Robustness

The robustness of the developed method was verified by doing minor changes in chromatographic conditions such as composition of the mobile phase ($\pm$ 0.3%), flow rate of the mobile phase ($\pm$ 0.2 ml/min), wavelength ($\pm$ 3 nm), did not substantially affect peak shape and the retention time of the sample.

3.3.7 Stability

The spiked plasma samples were stable for I week under all of the conditions; room temperature (25°C), refrigerated (2 – 8°C) or frozen at (-80°C), while the standard solutions were stable for about a month when stored at 2 – 8°C.

4. Application of the method

The proposed method is a part of comprehensive clinical study on "Pharmacokinetic drug-drug interaction of meloxicam, 5-hydroxy meloxicam and 5-carboxy meloxicam with commonly co-prescribed drugs like omeprazole and fluconazole in healthy human volunteers". The developed method was successfully applied for the determination of meloxicam in healthy human volunteers. Pharmacokinetic data was analyzed by using PK summit® software and will be published somewhere else. In all the analyzed plasma, saliva samples both metabolites 5-hydroxy meloxicam and 5-carboxy meloxicam concentrations were below the detection limit and thus could not be quantified and were only quantified in urine. This method was applied to the ongoing clinical trial and can also be employed for the quantification of meloxicam in various pharmaceutical dosage form as well.

5. Conclusion

The present developed method was optimized and validated for the simultaneous determination of meloxicam, 5-hydroxy meloxicam and 5-carboxy meloxicam in human biological fluids. The method was simple, rapid, easy, accurate, precise and economical. Various chromatographic conditions were optimized for the analysis of these analytes according to standard guidelines. The effects of composition of the mobile phase, flow rate, temperature of the column oven, detection wavelength were studied. In this method meloxicam and its metabolites recovery was excellent from biological sample by using two step LLE procedures. All the peaks appear within 7 min in a single chromatographic run. This method can also be employed in various pharmaceutical dosage forms, in biological matrices and in pharmacokinetic studies.

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Fig. 2. The chromatogram of standard sample spiked with 100 ng/ml of meloxicam (peak -4), 75 ng/ml of each metabolite i.e 5-hydroxy meloxicam (peak-1); 5-carboxy meloxicam (peak-3) and 1000ng/ml of internal standard (peak-2).

Fig. 3. Calibration curves of (A) meloxicam, (B) 5-hydroxy meloxicam, (C) 5-carboxy meloxicam. Solid line (―) represents spiked plasma, dashed line (---) represents spiked urine, dotted line (….) represents spiked saliva.

Fig. 4. RP-HPLC chromatograms of different samples, Peak-1; 5-hydroxy meloxicam, 2; internal standard, 3; 5-carboxy meloxicam, 4; meloxicam. Chromatograms a; blank solvent, b; blank plasma, c; plasma sample spiked with 1000ng/ml of meloxicam, 500ng/ml of 5-hydroxy meloxicam, 500ng/ml of 5-carboxy meloxicam and 1000ng/ml of internal standard, d; plasma sample.

Fig. 5. RP-HPLC chromatograms of different samples, Peak-1; 5-hydroxy meloxicam, 2; internal standard, 3; 5-carboxy meloxicam, 4; meloxicam. Chromatograms a; blank solvent, b; blank urine, c; urine sample spiked with

1000ng/ml of meloxicam, 500ng/ml of 5-hydroxy meloxicam, 500ng/ml of 5-carboxy meloxicam and 1000ng/ml of internal standard, d; urine sample.

Fig. 6. RP-HPLC chromatograms of different samples, Peak-1; 5-hydroxy meloxicam, 2; internal standard, 3; 5-carboxy meloxicam, 4; meloxicam. Chromatograms a; blank solvent, b; blank saliva, c; saliva sample spiked with 1000ng/ml of meloxicam, 500ng/ml of 5-hydroxy meloxicam, 500ng/ml of 5-carboxy meloxicam and 1000ng/ml of internal standard, d; saliva sample.

Fig. 7. RP-HPLC chromatogram showing peaks of 5-hydroxy meloxicam (peak-1), 5-carboxy meloxicam (peak-3) and meloxicam (peak-4) at the lower limit of detection.

Fig. 8. RP-HPLC chromatogram showing peaks of 5-hydroxy meloxicam (peak-1), 5-carboxy meloxicam (peak-3) and meloxicam (peak-4) at the lower limit of quantification.

Table-2: Accuracy, linearity, calibration, sensitivity, repeatability of the current RP-HPLC method.

Parameters	Plasma			Urine			Saliva		
	Meloxicam	5-Hydroxy Meloxicam	5-Carboxy Meloxicam	Meloxicam	5-Hydroxy Meloxicam	5-Carboxy Meloxicam	Meloxicam	5-Hydroxy Meloxicam	5-Carboxy Meloxicam
Accuracy (% recovery)									
Spiked concentration 1[a] (mean ± SD)	99.12±0.219	97.24±0.311	95.54±0.298	98.51±0.372	99.66±0.421	96.48±0.408	97.85±0.335	92.86±0.317	94.32±0.368
Spiked concentration 2[a] (mean ± SD)	98.49±0.373	97.91±0.566	97.62±0.471	99.59±0.619	99.82±0.737	95.10±0.582	94.67±0.541	90.93±0.493	91.41±0.629
Spiked concentration 3[a] (mean ± SD)	98.83±0.529	96.75±0.758	98.11±0.883	99.08±0.944	99.32±0.895	93.98±0.817	95.09±0.786	94.78±0.655	92.23±0.904
Accuracy (% RSD)									
Spiked concentration 1[a]	0.70	0.59	0.54	0.81	0.69	0.58	0.84	0.72	0.62
Spiked concentration 2[a]	0.54	0.41	0.38	0.63	0.49	0.43	0.66	0.52	0.48
Spiked concentration 3[a]	0.38	0.29	0.32	0.41	0.33	0.38	0.47	0.43	0.42
Calibrations range (ng. mL^{-1})	10.0-2000.0	30.0-1000.0	25.0-1000.0	10-2000.0	30-1000.0	25.0-1000.0	10.0-2000.0	30.0-1000.0	25.0-1000.0
Linearity Spiked samples Slope (b) Intercept (a) Correlation-coefficient (r)	0.002 0.0102 0.9999	0.0006 0.0031 0.9997	0.0012 0.0015 0.9991	0.0002 0.0664 0.9996	0.0006 0.0138 0.9995	0.0011 0.0001 0.9997	0.0018 0.0477 0.9993	0.0005 0.0026 0.9997	0.001 0.0008 0.9996
Repeatability's Injection repeatability (mean; %RSD) Spiked concentration 3[b]	[c] 6.16.; 0.53	[c] 2.72; 0.33	[C] 4.36; 0.24	[c] 6.17.; 0.58	[c] 2.71; 0.36	[C] 4.37; 0.26	[c] 6.16.; 0.49	[c] 2.73; 0.29	[C] 4.36; 0.32
Spiked concentration 3[b]	[d]162652; 1.25	[d] 49475; 0.87	[d]79063; 0.93	[d]159548; 1.77	[d] 50342; 1.01	[d]74274; 0.96	[d]150548; 1.11	[d] 41231; 0.92	[d]69584; 1.17
Analysis repeatability (mean;%RSD) Spiked concentration 3[b]	[d]163084; 0.75	[d]49371; 0.41	[d]78269; 0.39	[d]160856; 0.91	[d]52597; 0.52	[d]76568; 0.44	[d]148357; 0.72	[d]40217; 0.47	[d]70482; 0.41
Sensitivity Lower limit of the detection, LLOD (ng.mL^{-1})	3.0	10.0	8.0	3.0	10.0	8.0	3.0	10.0	8.0
Lower limit of the quantification, LLOQ (ng.mL^{-1})	9.0	32.0	25.0	9.0	32.0	25.0	9.0	32.0	25.0

Spiked concentration 1= meloxicam: 50ng.mL^{-1}, 5-hydroxy meloxicam: 50ng.mL^{-1} and 5-carboxy meloxicam: 50ng.mL^{-1}; Spiked concentration 2 = meloxicam: 100 ng.mL^{-1}, 5-hydroxy meloxicam: 100ng.mL^{-1} and 5-carboxy meloxicam: 100ng.mL^{-1}; Spiked concentration 3 = meloxicam:1000ng.mL^{-1}, 5-hydroxy meloxicam: 1000ng.mL^{-1} and 5-carboxy meloxicam: 1000ng.mL^{-1}.[a] n= 5, [b]n= 10, [c]Retention time (min), [d]Peak area, [e] Amount recovered.

Table-2: The data of the Intra days and Inter days Precision (n = 3).

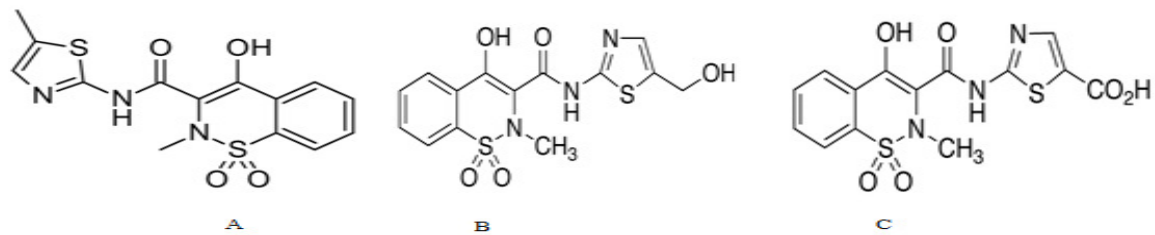| Concentration spiked (ng.mL⁻¹)	Plasma concentration(ng.mL⁻¹)				Urine concentration (ng.mL⁻¹)				Saliva concentration (ng.mL⁻¹)		
	Intra day (mean±S.D)	% RSD	Inter day (mean±S.D)	% RSD	Intra day (mean±S.D)	% RSD	Inter day (mean±S.D)	% RSD	Intra day (mean±S.D)	% RSD	Inter day (mean±S.D)
Meloxicam 50.0 100.0 2000.0	49.552±0.2607 98.286±0.4377 1979.658±2.8924	0.5261 0.4454 0.1461	49.297±0.3633 97.116±0.5965 1949.466±2.889	0.7369 0.6142 0.1482	49.258±0.3911 99.312±0.7243 1975.490±1.9962	0.7939 0.7293 0.1010	48.987±0.4521 98.045±0.8117 1952.656±2.5431	0.9228 0.8278 0.1302	48.729±0.4149 95.298±0.6980 1900.486±2.6626	0.8514 0.7324 0.1401	48.455±0.5654 94.513±0.9120 1896.724±2.7223
5-Hydroxy meloxicam 50.0 100.0 1000.0	48.698±0.2152 97.712±0.3078 966.274±1.0560	0.4419 0.3150 0.1093	48.127±0.4699 96.850±0.6999 960.839±1.4980	0.9763 0.7226 0.1559	49.696 ±0.3741 99.388±0.7816 995.449±0.9981	0.7525 0.7864 0.1002	49.258 ±0.5494 99.131±0.9146 990.507±2.3118	1.1153 0.9226 0.2333	46.578 ±0.2376 93.319±0.4649 949.996±2.9781	0.5101 0.4981 0.3134	45.824 ±0.1855 92.857±0.4963 942.383±2.3756
5-Carboxy meloxicam 50.0 100.0 1000.0	48.214±0.8190 96.035 ± 1.1503 973.050±2.6483	1.7018 1.1978 0.2721	47.755±0.8460 94.910±1.2569 968.464±1.4671	1.7715 1.3243 0.1515	48.037 ±0.4933 96.821 ± 0.8662 944.966±1.0892	1.0269 0.8946 0.1152	47.488 ±0.7372 96.114 ± 1.3742 940.339±1.4611	1.5523 0.8946 0.1152	47.328 ±0.5916 94.536 ± 0.7864 919.327±1.1232	1.2500 0.8318 0.1221	46.674 ±0.3117 93.288 ± 0.9833 913.407±1.3686

Figure 1: Structures; A) meloxicam, B) 5-hydroxy meloxicam, C) 5-carboxy meloxicam.
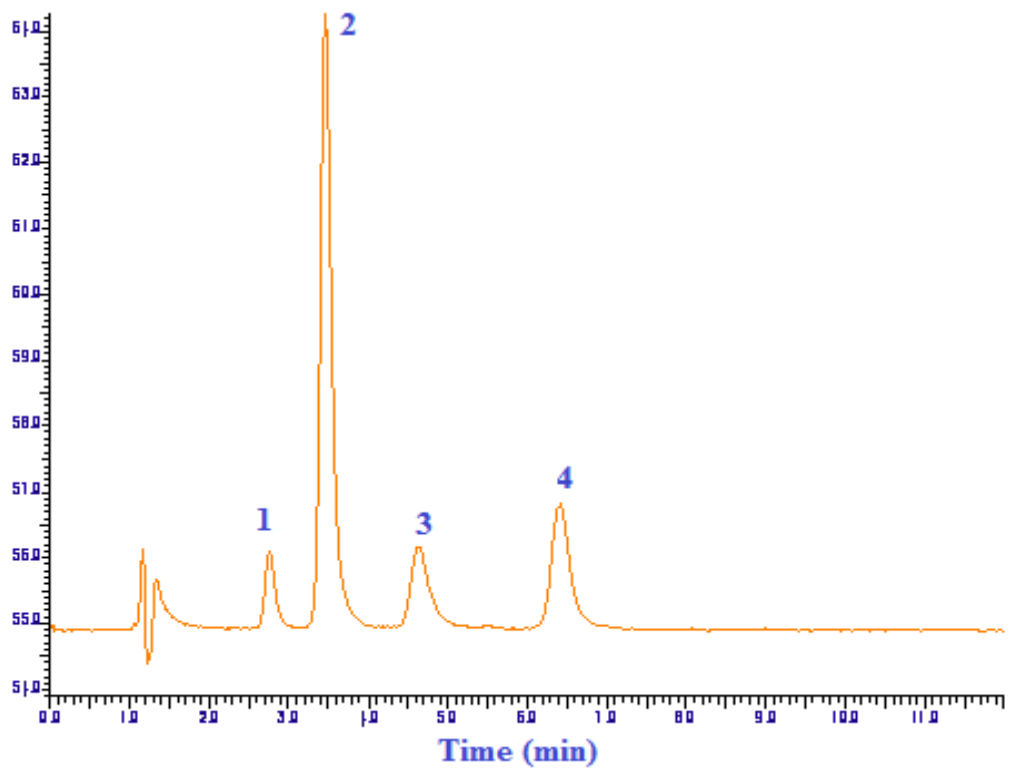
Figure 2: The chromatogram of standard sample spiked with 100 ng/ml of meloxicam (peak -4), 75 ng/ml of each metabolite i.e 5-hydroxy meloxicam (peak-1); 5-carboxy meloxicam (peak-3) and 1000ng/ml of internal standard (peak-2).
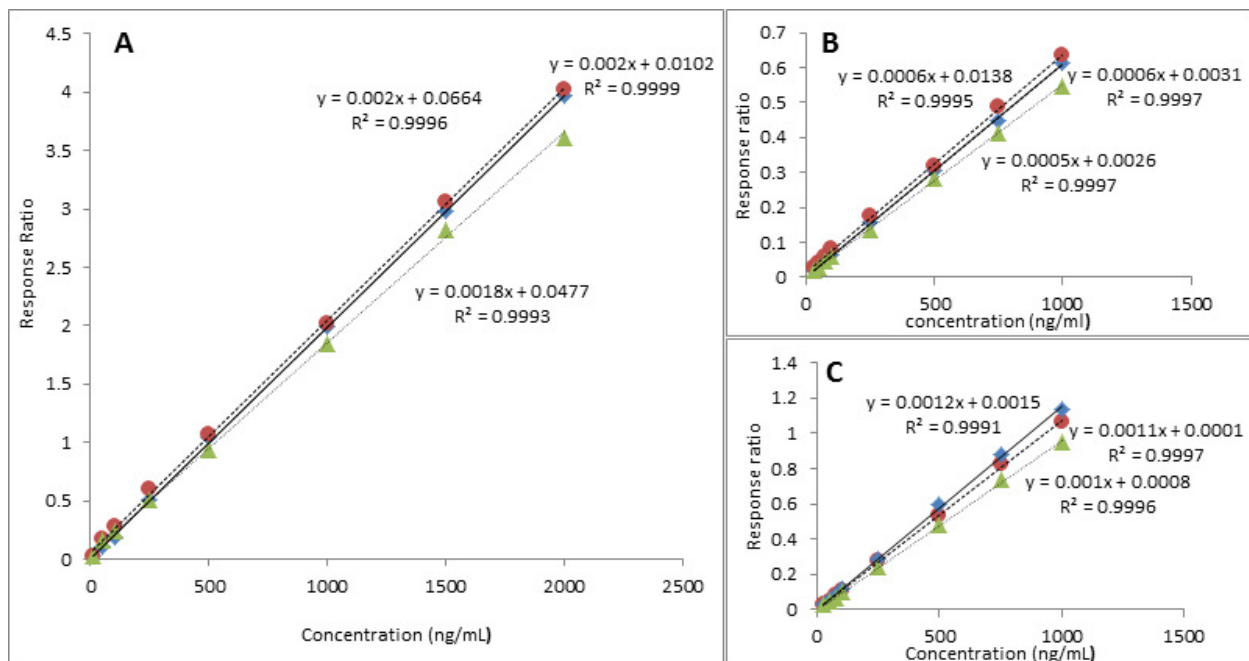
Figure 3: Calibration curves of (A) meloxicam, (B) 5-hydroxy meloxicam, (C) 5-carboxy meloxicam. Solid lines (—) represent spiked plasma, dashed lines (---) represent spiked urine and dotted lines (….) represent spiked saliva.
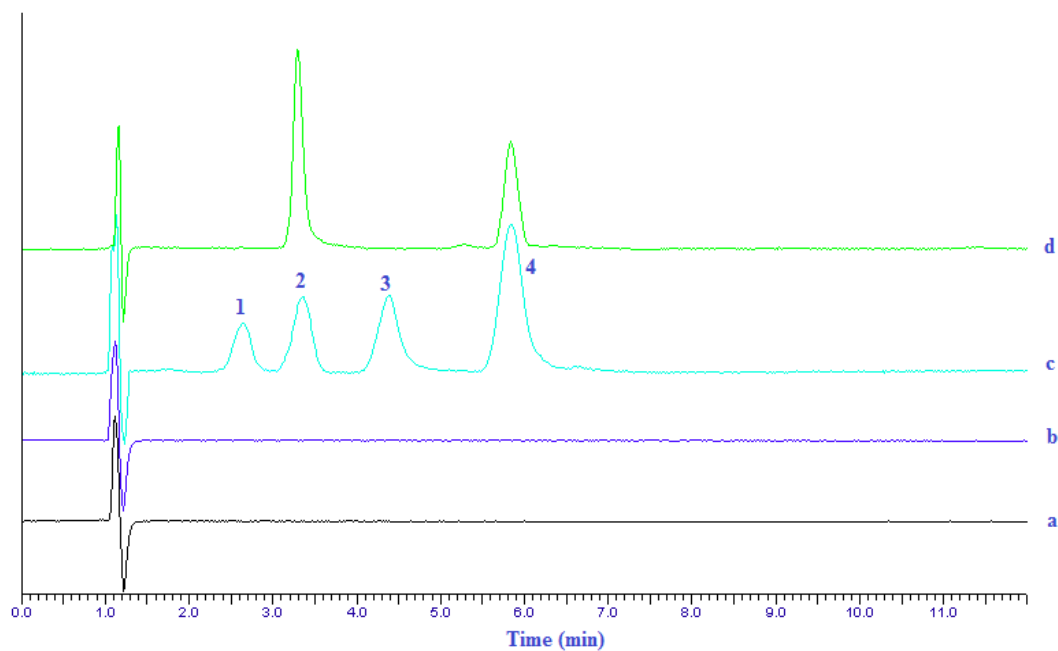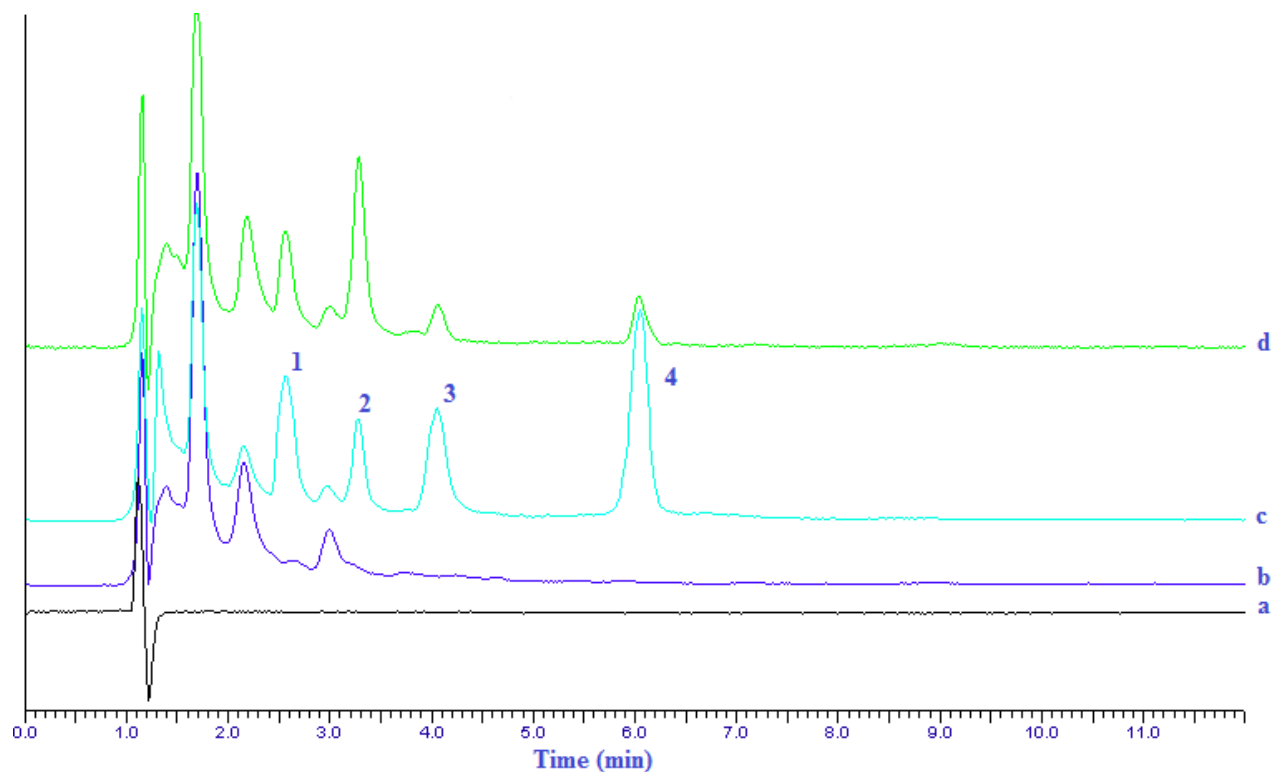
Figure 4: HPLC chromatogram's of various samples, Peak-1; 5-hydroxy meloxicam, 2; internal standard, 3; 5-carboxy meloxicam, 4; meloxicam. Chromatogram's a; blank-solvent, b; blank-plasma, c; plasma spiked sample with 1000ng/ml of meloxicam, 500ng/ml of 5-hydroxy meloxicam, 500ng/ml of 5-carboxy meloxicam and 1000ng/ml of internal standard, d; plasma sample.
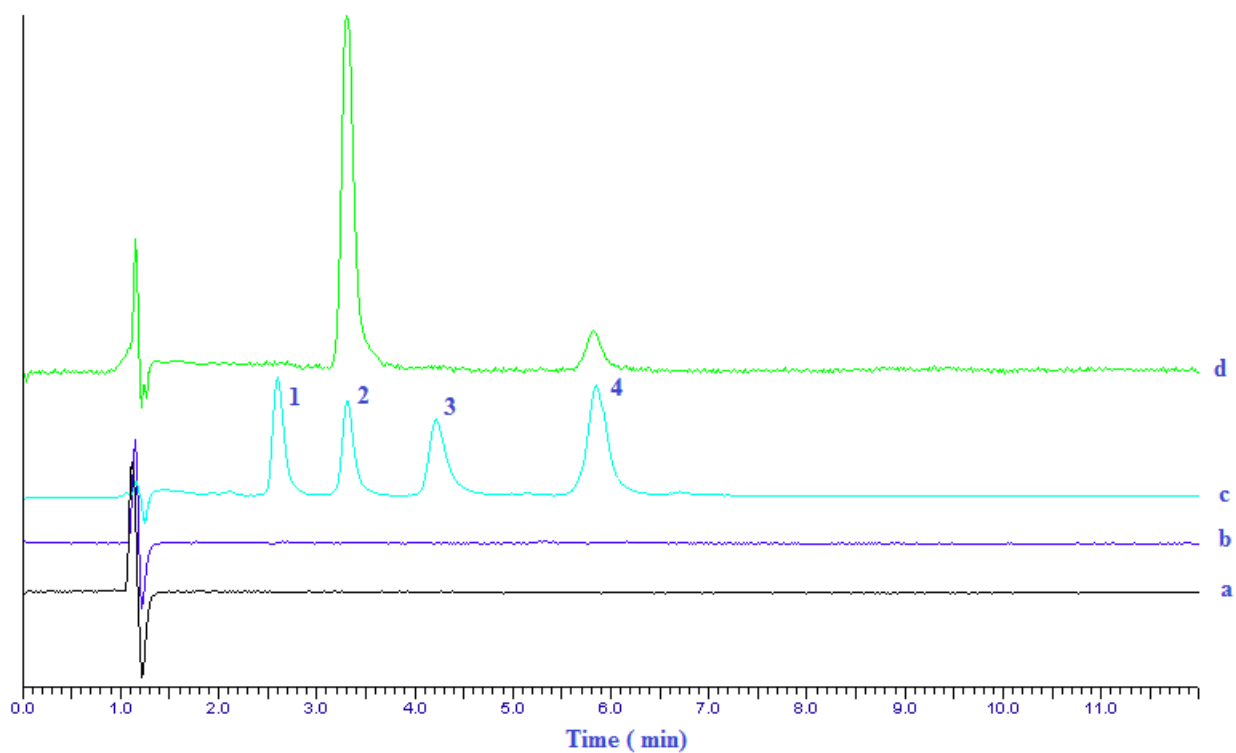
Figure 5: HPLC chromatogram's of various samples, Peak-1; 5-hydroxy meloxicam, 2; internal standard, 3; 5-carboxy meloxicam, 4; meloxicam. Chromatogram's a; blank-solvent, b; blank-urine, c; urine spiked sample with 1000ng/ml of meloxicam, 500ng/ml of 5-hydroxy meloxicam, 500ng/ml of 5-carboxy meloxicam and 1000ng/ml of internal standard, d; urine sample.

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Figure 6: HPLC chromatogram's of various samples, Peak-1; 5-hydroxy meloxicam, 2; internal standard, 3; 5-carboxy meloxicam, 4; meloxicam. Chromatogram's a; blank-solvent, b; blank-saliva, c; saliva spiked sample with 1000ng/ml of meloxicam, 500ng/ml of 5-hydroxy meloxicam, 500ng/ml of 5-carboxy meloxicam and 1000ng/ml of internal standard, d; saliva sample.
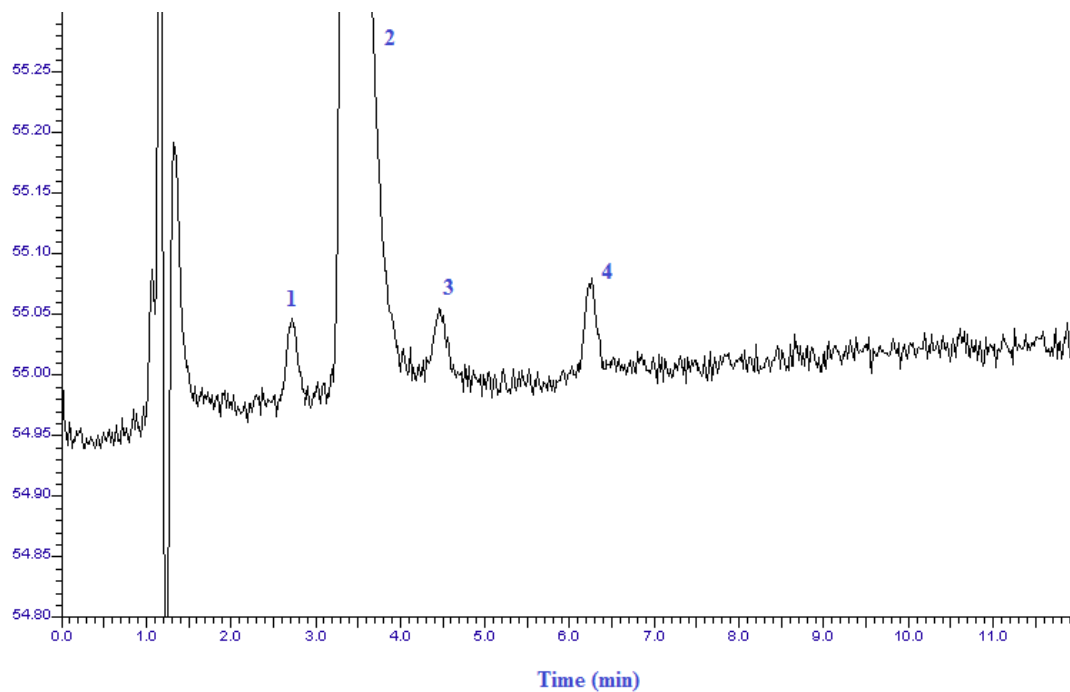
Figure 7: HPLC chromatogram showing peaks of 5-hydroxy meloxicam (peak-1), 5-carboxy meloxicam (peak-3) and meloxicam (peak-4) at the lower limit of detection.
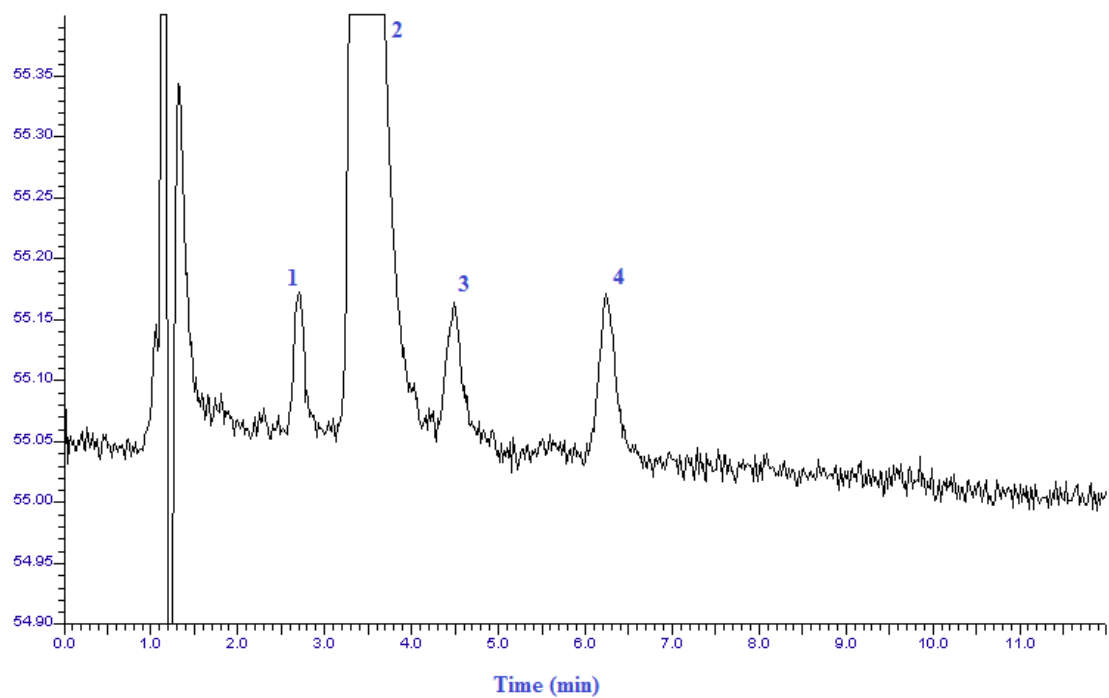
Figure 8: HPLC chromatogram showing peaks of 5-hydroxy meloxicam (peak-1), 5-carboxy meloxicam (peak-3) and meloxicam (peak-4) at the lower limit of quantification.