IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736. Volume 5, Issue 5 (Sep. – Oct. 2013), PP 17-24
www.iosrjournals.org

Haemolysis effect of Mefenamic Acid 250 mg Capsule in Bio analysis by liquid chromatography - tandem mass spectrometry

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Abstract: *A rapid, simple and specific method for estimation of Mefenamic acid in human plasma was validated using Indomethacin as internal standard. The analyte and internal standard were extracted from plasma using simple solid phase extraction. The compound were separated on a reverse-phase column with an isocratic mobile phase consisting of 2 mM Ammonium Acetate in Water and acetonitrile (20:80, v/v) and detected by tandem mass spectrometry in negative ion mode. The ion transition recorded in multiple reaction monitoring mode were m/z 240.1→ 196.0 for Mefenamic acid and m/z 356.1→312.0 for internal standard. Linearity in plasma was observed over the concentration range 35.000 – 7000.000 ng/mL for Mefenamic acid. The cv of the assay was 4.89 % to 5.98 % and accuracy was 99.36 to 102.20 % Intra and Interday respectively at LLOQ level. The validated method was applied to bioequivalence study of 250 mg Mefenamic acid in 28 healthy human volunteers. Total 50 samples from individual volunteers identified as Haemolyzed which were analyze initial and repeat again to cross check the method reproducibity for Haeamolysis effect and compared which found acceptable range.*

Keywords : *Mefenamic acid; Indomethacin; LC-MS-MS; human plasma*

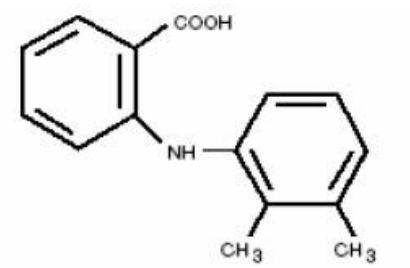
I. INTRODUCTION

In BA/BE studies Haemolysis effect can be accountable for analysis of drug estimation in plasma. Validation of LC-MS/MS assays includes an assessment of Haemolysis effects though the experiment. Identification of this effect in presence of drug can be helpful tool for estimation of assay in plasma during the course of subject analysis. Validation of the method to evaluate Haemolysis effect can easily prove the impact on drug estimation. Validation of drug estimation can be challenged through the Haemolysis samples which are unknown though Incurred sample reanalysis to compare the method ruggedness with respect to Haemolysis sample which are extracted directly from subjects (volunteers). Methodology can be applied to reduce Haemolysis effect through proper extraction techniques like SPE. We establish method with proper extraction techniques to reduce the Haemolysis effect and then challenge our method in subject analysis specifically for Haemolysis samples along with normal plasma samples. Summarize the assay value for combination drugs for Haemolysis samples and through ISR cross check the techniques used for validation is properly applied.

Mefenamic acid is a non-steroidal anti-inflammatory drug used to treat pain, including menstrual pain. It is typically prescribed for oral administration. Mefenamic acid decreases inflammation (swelling) and uterine contractions by a still unknown mechanism. However it is thought to be related to the inhibition of prostaglandin synthesis. There is also evidence that supports the use of Mefenamic acid for perimenstrual migraine headache prophylaxis, with treatment starting 2 days prior to the onset of flow or 1 day prior to the expected onset of the headache and continuing for the duration of menstruation.

Since hepatic metabolism plays a significant role in Mefenamic acid elimination, patients with known liver deficiency may be prescribed lower doses. Kidney deficiency may also cause accumulation of the drug and its metabolites in the excretory system. Therefore patients suffering from renal conditions should not be prescribed Mefenamic acid. It's rapidly absorbed. T max is 2 to 4 h. C max is 20 mcg/mL (single doses). Steady state is reached in 2 days and Apparent Vd is 1.06 L/kg. Protein binding is more than 90%. Excreted in breast milk. Metabolized by CYP-450 enzyme CYP2C9 to 3-hydroxymethyl Mefenamic acid (metabolite I). Further oxidation to a 3-carboxyMefenamic acid (metabolite II) may occur. The metabolites may undergo glucuronidation, and Mefenamic acid is also glucuronidated directly. Approximately 52% of a dose is excreted into the urine primarily as glucuronides of Mefenamic acid (6%), 3-hydroxyMefenamic acid (25%), and 3-carboxyMefenamic acid (21%). The fecal route of elimination accounts for up to 20% of the dose, mainly in the form of unconjugated 3-carboxyMefenamic acid. The half-life is approximately 2 h.

MEFENAMIC ACID is a member of the fenamate group of nonsteroidal anti-inflammatory drugs (NSAIDs). Each blue-banded, Mefenamic acid is a white to greyish-white, odorless, microcrystalline powder with a melting point of 230°-231°C and water solubility of 0.004% at pH 7.1. The chemical name is N-2,3-xylylanthranilic acid. The molecular weight is 241.29. Its molecular formula is C15H15N02 and the structural formula of Mefenamic acid is



Each capsule also contains lactose, NF. The capsule shell and/or band contains citric acid, USP; D&C yellow No. 10; FD&C blue No. 1; FD&C red No. 3; FD&C yellow No. 6; gelatin, NF; glycerol monooleate; silicon dioxide, NF; sodium benzoate, NF; sodium lauryl sulfate, NF; titanium dioxide, USP.

Several Method was reported to determine Mefenamic acid in plasma and serum. One of the method use diclofenac as IS and column as Thermo Hypurity C_{18}, 50 × 4.6 mm, 5 µm column with a mobile phase consisting of 2 m M ammonium acetate buffer and methanol (pH 4.5 adjusted with glacial acetic acid; 15:85, v/v) at a flow-rate of 0.75 mL/min and the total run time was 1.75 min. Analyte was introduced to the LC-MS/MS using an atmospheric pressure ionization source. Both the drug and IS were detected in negative-ion mode using multiple reaction monitoring *m/z* 240.0 → 196.3 and *m/z* 294.0 → 250.2.(1)

One of the stability studies was perform through RP-HPLC method for the determination of Mefenamic acid in raw material, different pharmaceutical forms, and in human serum for routine analysis, therapeutic purposes, and stability studies was developed and validated. The method developed constitutes mobile phase, acetonitrile: acetic acid: water (72.5:1:26.5, v/v/v) at pH 3 and Mefenamic acid was monitored with UV detection at 279 nm, eluting out at 3.98 min. The present HPLC method was found to be linear (100–300 µg mL−1), accurate. The limits of detection of the method were found to be 10 µg mL−1. (2)

Mefenamic acid with Indomethacin was determined with method used on HPLC through C18 column (250 x 4.6 mm I.D.) using 10 mM phosphoric acid-acetonitrile (40:60, v/v) as the mobile phase and both drugs were detected at 280 nm. The calibration graphs were linear with a correlation coefficient (r) of 0.999 or better from 0.1 to 10 micrograms/ml and the detection limits were 0.06 micrograms/ml for Indomethacin and 0.08 micrograms/ml for Mefenamic acid, for 50-microliters plasma samples.

In the present study a simple, reliable and rapid method have been developed and validated to estimate Mefenamic Acid in human plasma, with an advantage of simple Solid Phase Extraction method which allows for a high throughput of over 200 samples per day. SPE technique has been applied which ensured a lot more sample clean up resulting in better selectivity and higher recovery. The internal standard used was Indomethacin.

Total number of 20 samples of different time points has been collected with individual subjects in each period. Blood samples will be collected in Na- Heparin Vaccutainer. All the blood samples will be centrifuged under refrigeration with the machine set at, RPM, 10 minutes and 5°C. Plasma samples will be placed in deep freezer maintained at -70°C ± 10°C.

II. Experimental

Materials and chemicals

Reference standards of Mefenamic acid and Indomethacin were obtained from CHENG FONG CHEMICAL CO**.**, LTD and IFIS - FAB8RlCA Italian SINTETICI SpA respectively**.** These standards had purity ≥ 99 %. HPLC grade methanol and acetonitrile were purchased from J.T. Baker INC (Phillipsburg, NJ, USA). Ammonium Acetate of HPLC grade was procured from Merck Ltd (Mumbai, India). Phenomenex Strata-X (30 mg/1mL) SPE cartridges were procured from Orochem India Pvt. Ltd., (Mumbai, India). HPLC grade Water was used in the entire analysis.

Liquid chromatography and mass spectrometric conditions

The liquid chromatography system coupled with mass spectrometer (Applied Biosystems/API 2000) and temperature controlled compartment for column was used. The analytical column, Hypurity C18 (50 mm x 4.6 mm, 5 µm particle size) from Thermo Electron Corporation (Cheshire, UK) was used for separation of analyte and internal standard. Mobile phase of 2 mM Ammonium Acetate in Water with acetonitrile in ratio of 20:80 (v/v) was pumped isocratically at flow rate of 0.4 mL/min with 60% splitting. Auto sampler temperature was set at 10 °C and the injection volume was 3µL. The column oven temperature was maintained at 25 °C and the total LC run time was 1.38.

The Applied Biosystems/API 2000 LC-MS-MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 240.1 to the product ion m/z 196.0 for Mefenamic acid and the transition of the protonated molecular ion m/z 356.1 to the

product ion m/z 312.0 for the internal standard, Indomethacin. The instrument response was optimized for Mefenamic acid and Indomethacin by infusing a constant flow of a solution of the drug dissolved in mobile phase. Electrospray ionization (ESI) was performed in the Negative ion mode. (Table 1)

Preparation of standards and quality control samples

Two separate stock solutions of Mefenamic acid were prepared for bulk spiking of calibration curve and quality control samples for the method validation experiment as well as the subject sample analysis. A 1mg /mL stock solution for Mefenamic acid and Indomethacin were prepared by dissolving their accurately weighted compounds in methanol. This stock solution of Mefenamic acid thus prepared was serially diluted to prepare working solution in required concentration range with diluents methanol: water (70:30, v/v). The calibration standards and quality control (QC) samples were prepared by spiking (5 % of the total plasma volume) with working solutions. Calibration standards were prepared at concentration of 35, 70, 210, 700, 1400, 2800, 4200, 5600, and 7000 ng /mL for Mefenamic acid. Similarly, quality control standards (QC's) were prepared at four different concentrations namely, 35.700 (LLOQ), 98.000(LQC), 2450.000 (MQC) and 4900.00 (HQC) ng /mL. Sufficient calibration standards and quality control standards were prepared to validate the method and to serve as standards and controls during the assay of all study samples. However during the study, only three levels of controls were prepared as LQC (Lower Quality Control), MQC (Middle Quality Control) and HQC (Higher Quality Control). Aliquots of the standards and quality controls were stored together with the study samples at -70 °C until used for sample processing.

Extraction procedure

The plasma samples (100 µl) were transferred to 1.7-mL clear tubes (Tarsons, India) added 25µl of internal standard (working solution of 40.000 µg/mL of Indomethacin). The samples were vortexes to mix. 100 µL of 2.5 % (v/v) Ortho Phosphoric Acid in Water. The samples were vortexes for 30 sec. and centrifuged for 5 minutes at 14000 rpm at 10°C.. After centrifugation the samples were loaded on Phenomenex Strata-X (30 mg/1mL) cartridge pre-conditioned with 1 mL methanol followed by 1 mL of deionised water. The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The extraction cartridges washed with 1 mL of 10 % (v/v) of Methanol in Water (Twice). The analyte and the internal standard were eluted with 1.5 mL of elution solution; Mobile Phase. A 3 µL of the eluant was injected into the LC-MS-MS system through the autosampler.

III. Method validation

Selectivity:

Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, two Haemolyzed and two lipemic. They were processed as per the extraction method and their response was assessed at the retention time of analytes and the internal standard with six LLOQ samples for Mefenamic acid were prepared from the screened blank plasma samples which had the least interference.

Carry over:

Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, LLOQ, upper limit of quantitation (ULOQ) followed by blank plasma to check for any possible interference due to carryover.

Linearity and lower limit of quantification:

The linearity of the method was determined by analysis of five standard plots associated with a nine-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted (1/x2) linear regression. The calculation was based on the peak area ratio of analyte versus the area of internal standard. The concentration of the analyte were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor (1/x2). Several regression types were tested and the linear regression (weighted with 1/concentration2) was found to be the simplest regression, giving the best results ($r2 \geq 0.9985$).The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0% for LLOQ it should not be more than ±20.0%.

Accuracy and precision:

The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at

each quality control level were analyzed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (% CV) respectively.

Matrix effect:

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in replicate (n=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found were well within the acceptable limit set i.e. the RSD of area ratio to be within ± 15% at each level tested. Also, the ion suppression/ enhancement of analyte signal due to endogenous matrix interferences does not affect the quantification of analyte and IS peak which was confirmed by post-column infusion experiment. A standard solution containing Mefenamic acid (at MQC level) and IS was infused post column via a 'T' connector into the mobile phase at 10µL/min employing in-built infusion pump. Aliquots of 3µL of extracted control plasma were then injected into the column by the autosampler and MRM LC-MS/MS chromatogram was acquired for analytes. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of analyte would indicate ion enhancement.

Recovery:

Absolute recoveries of the analyte were determined at the three different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the un extracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples) representing 100% recovery.

Dilution integrity:

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼th concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

Stability:

All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against comparison samples of identical concentration. Stock solutions of Mefenamic acid and IS were checked for short term stability at room temperature and long term stability at 2-8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Bench top stability, autosampler stability (process stability), freeze thaw stability, and long-term stability in plasma were performed at LQC and HQC level using six replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at -20°C) and thawing (without warming) at room temperature. To meet the acceptance criteria, the difference between the stability and fresh samples should be within ±15%.

The same method was applied for pharmacokinetic study as A Randomized, Open Label, Balanced, Two-Treatment, Four-Period, Two-Sequence, Replicate, Crossover, Single Dose, Bioequivalence Study of Mefenamic Acid Capsule, USP 250 mg in Normal, Healthy, Adult, Human Subjects Under Fasting Condition

IV. FIGURES AND TABLES

TABLE 1: MASS PARAMETERS AND SOURCE PARAMETER

Mass parameters	Scan type	Parent ion	Product ion (m/z)	Dwell (msec)	DP	FP	EP	CE	CXP
Mefenamic Acid	MRM	240.1	196.0	200.0	-21.0	-260.0	-6.5	-22.0	-16.0
IS (Indomethacin)	MRM	356.1	312.0	200.0	-15.0	-360.0	-3.0	-11.0	-28.0

Source Parameters					
Ion Spray Voltage	**CUR**	**GAS 1**	**GAS 2**	**Temperature**	**CAD GAS**
-2000	25	30	75	400°C	3

TABLE 2: INTRA & INTER -BATCH PRECISION AND ACCURACY (*N*=6) OF MEFENAMIC ACID IN HUMAN PLASMA

Intra-batch precision and accuracy

Sr. No.	Run ID	Back Calculated Concentration (ng/mL)			
		LLOQ QC	LQC	MQC	HQC
Nominal Concentration (ng/mL)		**35.700**	**98.000**	**2450.000**	**4900.000**
1	051209_PA01	35.623	98.637	2452.441	4667.052
		37.450	98.521	2454.844	4793.923
		35.557	92.996	2395.119	4939.170
		36.892	95.350	2434.503	4848.495
		32.546	93.096	2386.585	4831.053
		34.768	93.368	2340.433	4788.612
Mean		**35.473**	**95.328**	**2410.654**	**4811.384**
SD		**1.733**	**2.661**	**44.765**	**89.161**
% CV		**4.89**	**2.79**	**1.86**	**1.85**
% Accuracy		**99.36**	**97.27**	**98.39**	**98.19**
N		**6**	**6**	**6**	**6**

Inter-batch precision and accuracy

Sr. No.	Run ID	Back Calculated Concentration (ng/mL)			
		LLOQ QC	LQC	MQC	HQC
Nominal Concentration (ng/mL)		**35.700**	**98.000**	**2450.000**	**4900.000**
1	051209_PA01	35.623	98.637	2452.441	4667.052
		37.450	98.521	2454.844	4793.923
		35.557	92.996	2395.119	4939.170
		36.892	95.350	2434.503	4848.495
		32.546	93.096	2386.585	4831.053
		34.768	93.368	2340.433	4788.612
2	071209_PA02_ABSOLUTE RECOVERY	41.795	105.090	2548.552	5213.232
		38.854	102.430	2598.269	5183.281
		35.366	111.151	2481.138	5003.893
		37.973	99.241	2508.364	4997.031
		38.391	100.242	2509.805	5045.771
		37.459	103.983	2504.560	4861.015
3	081209_PA03	36.644	100.417	2421.351	4779.551
		34.726	99.497	2480.427	4805.122
		35.376	99.378	2391.362	4753.173
		33.580	104.975	2401.027	4725.976
		38.431	103.288	2468.122	4729.865
		35.310	101.746	2382.479	4774.446
Mean		**36.486**	**100.189**	**2453.299**	**4874.481**
SD		**2.183**	**4.714**	**66.070**	**156.533**
% CV		**5.98**	**4.71**	**2.69**	**3.21**
% Accuracy		**102.20**	**102.23**	**100.13**	**99.48**
N		**18**	**18**	**18**	**18**

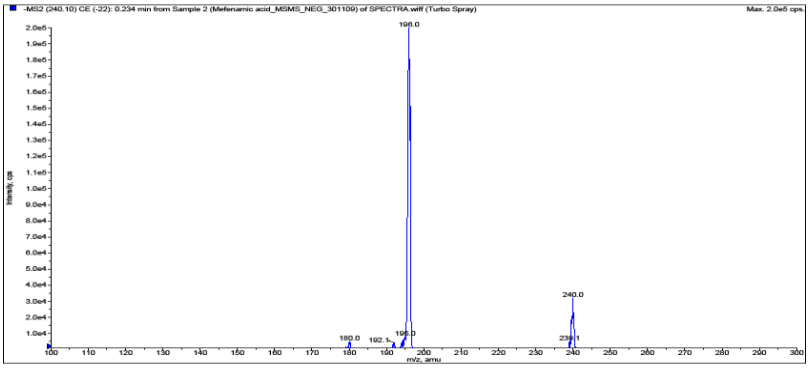
TABLE 1: INCURRED SUBJECT REANALYSIS RESULT OF HAEMOLYSIS SAMPLES FOR MEFENAMIC ACID

Sr. No.	Haemolyzed Sample I.D	Initial Conc. (ng/mL) (A)	Repeat Conc.(ng/mL) (B)	Mean C:(A+B)/2	% Diff
1	14 / I / 010	2139.385	2228.558	2183.972	4.08
2	14 / I / 019	161.862	161.264	161.563	-0.37
3	14 / II / 003	1455.672	1562.859	1509.266	7.10
4	14 / II / 020	110.755	116.372	113.564	4.95
5	14 / III / 014	1244.601	1334.404	1289.503	6.96
6	14 / III / 018	224.321	233.948	229.135	4.20
7	14 / IV / 005	2247.164	2416.615	2331.890	7.27

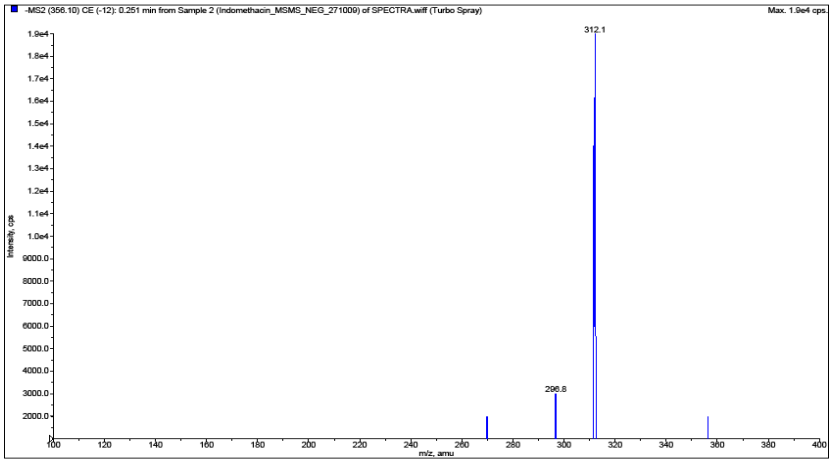
8	14 / IV / 019	139.159	143.816	141.488	3.29
9	15 / I / 006	1803.310	1679.761	1741.536	-7.09
10	15 / I / 020	171.359	179.986	175.673	4.91
11	15 / II / 012	1057.253	1101.129	1079.191	4.07
12	15 / II / 019	168.905	183.351	176.128	8.20
13	15 / III / 004	1441.040	1430.817	1435.929	-0.71
14	15 / III / 020	175.363	176.998	176.181	0.93
15	15 / IV / 015	1348.876	1266.435	1307.656	-6.30
16	15 / IV / 019	207.021	206.141	206.581	-0.43
17	16 / I / 008	1495.969	1543.760	1519.865	3.14
18	16 / I / 020	132.879	154.740	143.810	15.20
19	16 / II / 012	1403.765	1355.912	1379.839	-3.47
20	16 / II / 020	232.369	225.398	228.884	-3.05
21	16 / III / 006	1507.902	1444.122	1476.012	-4.32
22	16 / III / 019	147.371	155.882	151.627	5.61
23	16 / IV / 013	1301.764	1287.531	1294.648	-1.10
24	16 / IV / 020	242.118	228.385	235.252	-5.84
25	17 / I / 006	1629.717	1832.824	1731.271	11.73
26	17 / I / 018	155.115	168.777	161.946	8.44
27	17 / II / 006	2441.407	2399.879	2420.643	-1.72
28	17 / II / 019	115.525	125.973	120.749	8.65
29	17 / III / 004	2927.379	2970.130	2948.755	1.45
30	17 / III / 017	128.528	135.021	131.775	4.93
31	17 / IV / 006	1174.707	1155.573	1165.140	-1.64
32	17 / IV / 017	138.361	143.802	141.082	3.86
33	18 / I / 013	1878.828	2027.361	1953.095	7.61
34	18 / I / 020	138.677	157.105	147.891	12.46
35	18 / II / 004	2096.256	2206.808	2151.532	5.14
36	18 / II / 020	114.321	122.054	118.188	6.54
37	18 / III / 012	1663.814	2088.700	1876.257	22.65
38	18 / III / 019	160.808	224.615	192.712	33.11
39	18 / IV / 005	3194.417	3713.277	3453.847	15.02
40	18 / IV / 019	128.533	153.192	140.863	17.51
41	19 / I / 005	1815.082	1821.016	1818.049	0.33
42	19 / I / 019	130.712	134.776	132.744	3.06
43	19 / II / 006	3583.209	3680.071	3631.640	2.67
44	19 / II / 019	127.208	129.712	128.460	1.95
45	19 / III / 006	2449.031	2288.346	2368.689	-6.78
46	19 / III / 019	133.922	131.513	132.718	-1.82
47	19 / IV / 018	128.220	118.301	123.261	-8.05
48	20 / II / 010	723.307	733.472	728.390	1.40
49	20 / III / 013	214.162	228.143	221.153	6.32
50	20 / IV / 019	199.991	248.047	224.019	21.45

FIGURE 1: PRODUCT ION MASS SPECTRA OF MEFENAMIC ACID AND INDOMETHACIN

Mefenamic Acid



INDOMETHACIN



V. RESULT AND DISCUSSION

The mean absolute recoveries of Mefenamic acid determined at 98.000, 2450.000 and 4900.000 ng /mL were 81.06(RSD 6.04%), 83.28(RSD 1.71%) and 87.73 % (RSD 1.80%), respectively. The mean absolute recovery of Indomethacin was 92.84 %(RSD 1.3 %).Minimum matrix effect for Mefenamic acid was observed from the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 2.07 % for Mefenamic acid. For the internal standard the RSD of the area ratios over both LQC and HQC levels was less than 4.4 %.This indicated that the extracts were "clean" with no co-eluting compounds influencing the ionization of the analyte and the internal standard.

The high selectivity of MS-MS detection allowed the development of a very specific and rapid method for the determination of Mefenamic acid in plasma. Representative chromatograms obtained from blank plasma and blank plasma spiked with LLOQ standard is presented. No significant interfering peak of endogenous compounds was observed at the retention time of analyte in blank human plasma containing Na Heparin as the anti-coagulant in ten different plasma lots which was compared versus six replicates of extracted samples at the LLOQ level.

The LLOQ, defined as that concentration of Mefenamic acid which can still be determined with acceptable precision (%RSD < 20) and accuracy (bias within ± 20%) was found to be 25 ng /mL. Results of the intra-batch and inter-batch validation assays are presented in Tables 2, respectively. The inter-batch and intra-batch precision were ≤ 5.98 % and ≤ 4.89 % whereas the inter-batch and intra-batch accuracy in terms of % bias were within the range of 2.23 to -0.52 and -2.73 to 0.64 for Mefenamic acid respectively.

Bench top and processed (autosampler) stability for Mefenamic acid were performed at LQC and HQC levels. The results revealed that Mefenamic acid was stable in plasma for at least 13 h at room temperature. It was confirmed that repeated freeze and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of Mefenamic acid. Mefenamic acid was found stable for minimum five freeze and thaw cycles. The long term stability results also indicated that Mefenamic acid was stable in human plasma for up to 94 days at a storage temperature of -20 °C. This period of long term stability was sufficient enough to cover the entire storage period from first day of storage of the plasma samples to the last day of analysis.

During method development different options were evaluated to optimize sample extraction, detection parameters and chromatography. Electrospray ionization (ESI) was evaluated to get better response of analytes as compared to atmospheric pressure chemical ionization (APCI) mode. In the nonionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient clean up. Best signal for the analyte was achieved with the ESI Negative ion mode. A mobile phase containing buffer ammonium acetate and format salt at different molarity and acetonitrile in varying combinations was tried during the initial development stages. The effect of pH of buffer also checked on sensitivity and peak shape. But the best signal and peak shape for Mefenamic acid was achieved using a mobile phase 2 mM Ammonium Acetate in Water in deionised water in combination with acetonitrile (20: 80 v/v). Use of a short Hypurity C18 (50 mm x 4.6 mm, 5µ) column resulted in reduced flow rate (0.4 ml/min,60% splitting) and reduced run time. The retention times for Mefenamic acid and Indomethacin were ~ 1.38 minutes and ~ 1.33 minutes, respectively. Product ions were identified during tuning and 196 m/z amu and 312 m/z amu. (Figure 1)

Indomethacin used as internal standard belonged to the same therapeutic category as Mefenamic acid. Ionization, retention and extraction characteristics were found to be similar to that of Mefenamic acid and hence it was selected as the internal standard of choice.

VI. Conclusion

A simple, selective and rapid method for the estimation of Mefenamic acid in human plasma was developed and validated, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The validated method was used in a cross over bioequivalence study in which 28 healthy volunteers were enrolled; each subject dosed 250 mg Mefenamic acid capsule (reference and test) as a two oral dose. Whereas LLOQ of 25 ng /mL. The method allows higher sample throughput due to the short chromatography time and simple sample preparation. Robust LC-MS-MS performance was observed, with acceptable variation in instrument response within batches. This method is an excellent analytical option for rapid quantification of Mefenamic acid in human plasma. Result shown in table 3 with initial run while Sample Analysis compare with Incurred subject re analysis to demonstrate method reproducibility as 94 % ISR samples meet acceptance criteria for Mefenamic acid .whereas the acceptance criteria for ISR is 67% samples should meet + or – 20% difference.

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