

Determination of Amtolmetin and Its Active Metabolites in Plasma by HPLC-UV: Application to a Bioequivalence Study

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Abstract

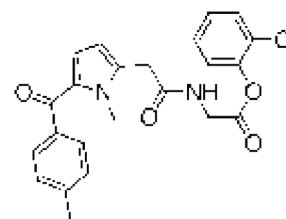
A simple, rapid and selective method was developed for the determination of amtolmetin guacil, tolmetin sodium and tolmetin glycinamide from human plasma. The method involves extracting amtolmetin guacil, tolmetin sodium and tolmetin glycinamide with acetonitrile using coumarin as internal standard. Chromatographic separation was carried out on a C8 column using mixture of acetonitrile:methanol:1% acetic acid as mobile phase with UV detection set at 313 nm. The retention time of AG, T, TG and IS were 8.20 ± 0.2 , 5.3 ± 0.2 , 4.0 ± 0.2 and 4.9 ± 0.2 min, respectively. The method was validated and found to be linear in the range of 0.5-20.0 $\mu\text{g/ml}$ for amtolmetin guacil, tolmetin sodium and tolmetin glycinamide. The co-efficient of variation for intra-day and inter-day accuracy and precision was $< 8.2\%$ for amtolmetin guacil, tolmetin sodium and tolmetin glycinamide. An open, randomized, two-treatment, two period, single dose crossover, bioequivalence study in twelve fasting, healthy, male, volunteers was conducted. After dosing, serial blood samples were collected for the period of 24 h. Various pharmacokinetic parameters for both the active metabolites (tolmetin and tolmetin glycinamide) were determined from plasma concentration of both formulations. Log transformed values were compared by analysis of variance (ANOVA) followed by classical 90% confidence interval for C_{max} , AUC_{0-t} and $AUC_{0-\text{inf}}$ for both the active metabolites (tolmetin and tolmetin glycinamide) and it was found that both test and reference products were bioequivalent. The proposed method proved to be rapid, precise and accurate and can be successfully used in a bioequivalence study of amtolmetin guacil tablet.

Keywords: Amtolmetin guacil; Tolmetin; Tolmetin glycinamide; Bioequivalence; HPLC-UV

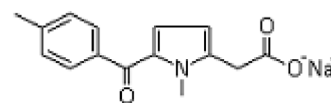
Introduction

Amtolmetin guacil (AG) (Figure 1) is a non-acidic prodrug of tolmetin, having similar NSAID properties like tolmetin with additional analgesic, antipyretic and gastroprotective properties. Amtolmetin is formed by amidation of tolmetin by glycine. Amtolmetin guacil is hydrolysed in the gastrointestinal tract and is absorbed predominantly as tolmetin-glycinamide (TG). TG is metabolized to tolmetin (T) in the tissues, which is then metabolized to methyl carboxybenzoyl pyrrole acetic acid (MCPA). It also enhances the NOS activity of gastric mucosae, which facilitates the synthesis and release of NO so as to reduce the gastrointestinal system damage (Tubaro et al., 2000; Li et al., 2004; Lazzaroni et al., 2001). Several HPLC methods have been developed for the determination of amtolmetin and its metabolite.

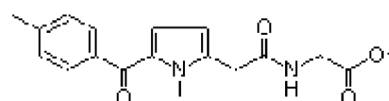
Mancinelli et al. (1991) described a method for the determination of amtolmetin and its metabolite from rats plasma. Although the method is quite sensitive it requires a gradient HPLC along with the liquid-liquid extraction which is time consuming and also the solvent used for extraction are well known carcinogens, which may be difficult in handling as lot of samples analysed for a bioequivalence study. Annunziato and di Renzo, (1993) described a method for the analysis of AG for a bioequivalence of two 600 mg AG capsules. (Only abstract is available as the method literature is published in Italian language). The other reported methods for determination of tolmetin (one of the active metabolite of amtolmetin) and its metabolites,



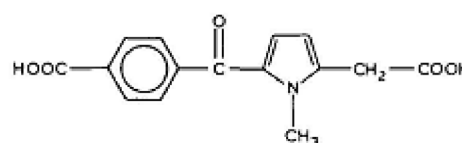
Amtolmetin Guacil



Tolmetin Sodium



Tolmetin-glycinamide



Methyl Carboxybenzoyl Pyrrole Acetic acid

Figure 1: Chemical structure of AG, T, TG and MCPA.

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describe the use of either an ion-pairing chromatographic method or multi-step sample preparation techniques along with Rp-2 column (Hyneck et al., 1987; Desiraju et al., 1982).

This report describes the development and validation of a simple and rapid HPLC assay for the simultaneous determination of amtolmetin and its two active metabolites in human plasma, which can be routinely applied for the analysis of bioequivalence study samples.

Materials and Methods

Chemicals and reagents

Amtolmetin guacil (AG), tolmetin sodium (T), tolmetin glycinamide (TG) were obtained from Macleods Pharmaceuticals Ltd, Mumbai, India. Coumarin was obtained from Dr. M.K.R Drug Testing Laboratory, Mumbai. Acetonitrile and methanol (HPLC grade) were purchased from Qualigens Fine Chemicals, Mumbai. Acetic acid (AR grade) was purchased from S.D Fine Chem. Ltd., Mumbai. Freshly prepared double distilled water was used throughout the study. Fresh frozen human plasma used in the method development was obtained from the National Plasma Fractionation Center, K.E.M. Hospital, Mumbai, and was stored at -20°C until used.

Instrumentation and chromatographic conditions

The HPLC system consisted of a JASCO-PU 980 intelligent pump (JASCO Ltd., Japan), manual injector port with 20 μl loop (Rheodyne, USA) and JASCO UV-Vis 975 intelligent detector (JASCO Ltd., Japan). The wavelength of the detector was set at 313 nm. Detector output was quantified on JASCO-Borwin (Version 1.50) chromatography software with Hercules 2000 Chromatography Interface (Version 2.0). Separation was carried out on a Hiq Sil C8, 250 X 4.6 mm i.d., Japan, using acetonitrile:methanol:1% acetic acid in the ratio of 55:5:40 as a mobile phase, at a flow rate of 1 ml/min. The mobile phase was filtered through nylon membrane filter (0.45 μm pore size, Pall, Gelman Laboratories) and ultrasonically degassed prior to use. Total analysis time was 10 min. Data were obtained and processed on JASCO-Borwin (Version 1.50) chromatography software with Hercules 2000 chromatography Interface (Version 2.0).

Preparation of standard stock and working standard solutions

A stock standard solution of AG, T and TG were prepared separately in acetonitrile at a concentration of 1 mg/ml. 1 ml of this standard stock solution was further diluted with acetonitrile to 10 ml to yield 100 $\mu\text{g}/\text{ml}$ standard solution. Working standard solutions of AG, T and TG were prepared in the range of 0.25 to 20.0 $\mu\text{g}/\text{ml}$, by suitable dilution of the standard stock solution (100 $\mu\text{g}/\text{ml}$) with the mobile phase. These standard solutions were stored at 4°C .

Preparation of internal standard (IS) stock solution

A stock standard solution of 1 mg/ml coumarin was prepared in acetonitrile. 1 ml of this solution was further diluted with mobile phase to 10 ml yield 100 $\mu\text{g}/\text{ml}$ coumarin solution.

Preparation of standard solutions in plasma

Calibration standards in plasma were prepared by spiking drug

free plasma with 1 ml of 1000 $\mu\text{g}/\text{ml}$ standard stock solution (containing 1 mg/ml each of AG, T and TG) in a 10 ml volumetric flask. This solution was diluted to 10 ml with drug free plasma to furnish a calibration standard containing 100 $\mu\text{g}/\text{ml}$ each of AG, T and TG. 100 $\mu\text{g}/\text{ml}$ solution was further diluted with drug free plasma to furnish the calibration standards of 0.25, 0.50, 1.0, 2.5, 5.0, 10.0 and 20.0 $\mu\text{g}/\text{ml}$. They were frozen in small portions at -20°C till analyzed. The pH of the calibration standard solutions in plasma was adjusted to 3 with concentrated ortho phosphoric acid to prevent hydrolysis of metabolite.

Preparation of quality control standards in plasma

Quality control standards were prepared by spiking drug free plasma with standard solution containing AG, T and TG to furnish a 'Highest Quality Control standard' (HQC) 20 $\mu\text{g}/\text{ml}$, 'Median Quality Control standard' (MQC) 2.5 $\mu\text{g}/\text{ml}$ and 'Lowest Quality Control' standard (LQC) 0.25 $\mu\text{g}/\text{ml}$. The pH of these quality control standards in plasma was adjusted to 3 with concentrated ortho phosphoric acid. These samples were used to validate the method and were frozen in small portions at -20°C till analyzed.

Sample preparation

To 1 ml plasma sample containing AG, T and TG (calibration standards), 50 μl of an internal standard solution (100 $\mu\text{g}/\text{ml}$) was added and vortexed for 1 min. The drug and its active metabolite along with IS were extracted by vortexing the sample with 1.5 ml of acetonitrile for 30 s followed by centrifugation at 6000 g for 15 min at 4°C . 20 μl of the supernatant was injected onto the column.

Method validation

The analytical method developed for the determination of amtolmetin and its active metabolites from plasma was validated for its selectivity, limit of detection and quantitation, linearity, precision, accuracy, recovery and stability in plasma.

Selectivity

A quality control sample containing each of AG, T, TG (2.5 $\mu\text{g}/\text{ml}$) and internal standard was developed under the optimized chromatographic condition. The separation of AG, T, TG, IS and probable impurities from plasma was checked, by comparing the chromatogram of quality control sample with that of blank plasma chromatogram. Six drug free blank plasma samples were prepared and compared.

Limit of detection (LOD) and limit of quantitation (LOQ)

In order to estimate LOD and LOQ, the drug free blank plasma sample was extracted and injected six times and analysed as described under optimized chromatographic conditions. The noise level was then determined. Further drug spiked plasma was injected. The LOD [Signal-to-noise ratio = 3] and LOQ for AG, T and TG was determined [Signal-to-noise ratio = 10].

Linearity

Plasma samples were quantified using the ratio of the peak area of analytes to that of IS as the assay parameter. Peak area ratios were plotted against concentrations and the standard curves were in the form of $y = A + Bx$. The linearity of the method was evaluated by a calibration curve in the range of 0.25–20 $\mu\text{g}/\text{ml}$

for amlolmetin, and its metabolites in plasma including LOQ. For linearity study seven different concentrations of amlolmetin guacil, tolmetin sodium and tolmetin glycinamide were analyzed 0.25, 0.50, 1, 2.5, 5, 10 and 20 µg/ml. The calibration curve requirement was set at a correlation coefficient (r^2) of 0.99 or better.

Accuracy and precision

Intra-day and *inter-day* accuracy and precision were determined by analysis of quality control standards of AG, T and TG (0.25 µg/ml, 2.5 µg/ml, and 20 µg/ml), six times a day randomly and once on each of six different days respectively. Six samples of each concentration along with a fixed concentration of IS were developed under optimized chromatographic conditions and the response was measured. For acceptable *intra-day* and *inter-day* values, accuracy and precision should be within 85-115% and coefficient of variation (CV) values should be <15% respectively.

Recovery

The recoveries of AG, T and TG from plasma were determined on quality control standards (0.25 µg/ml, 2.5 µg/ml and 20 µg/ml) containing fixed amount of IS in each of these samples. Recoveries of AG, T and TG was determined by comparing each of the peak area ratios of extracted quality control standards with that of peak area ratio of unextracted standard solutions containing the corresponding concentrations of AG, T and TG in the mobile phase. Recoveries were performed in triplicate.

Stability studies

Stability of AG, T and TG in plasma was tested using quality control standards for three freeze-thaw cycle and long-term stabilities. In each freeze-thaw cycle, the quality control samples were frozen at -20°C for 24 h and thawed to room temperature. The long-term stability was evaluated by storing the quality control samples at -20°C over time and the concentration of AG, T and TG were found out on day 5, day 15 and day 30. The results were compared with those QC samples freshly prepared, and the percentage concentration deviation was calculated. For the acceptance criterion of stability, the deviation compared to the freshly prepared standard should be within ±15%.

Application to bioequivalence study

Clinical design

The developed and validated HPLC method was applied to determine the two active metabolites (i.e. tolmetin and tolmetin glycinamide) in a bioequivalence study of amlolmetin guacil in healthy Indian male volunteers with a mean age of 22.83 ± 1.59 years and a mean body mass index (BMI) of 21.98 ± 2.51 after a single dose of 600 mg amlolmetin guacil test and reference tablets. On the basis of medical history, clinical examination and laboratory investigation (hematology, blood biochemistry and urine analysis), healthy volunteers were selected for the study. The volunteers were instructed to abstain from taking any medication including over the counter (OTC) drugs for at least 2 weeks prior to and during the study period and avoid any alcohol or xanthine containing food and beverages 36 h prior to and during the course of the study. The study was approved by Independent Ethics Committee before obtaining written informed consent from all volunteers. The study was conducted accord-

ing to the principles outlined in the Declaration of Helsinki. The study was conducted as 12 x 2 single dose, randomized, open and complete crossover design. The volunteers fasted overnight before the study and for 4 h after the dosing. The volunteers received one tablet (test or reference) of amlolmetin guacil 600 mg with 240 ml water. Venous blood samples (5 ml) were collected before dosing (0 h) and then at 10 min, 20 min, 0.5, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00, 16.00, 20.00 and 24.00 h following drug administration, through an indwelling cannula into heparinised tubes. After drug administration standard breakfast and lunch were provided at 4 and 6 h, respectively. The blood samples collected at various time intervals after drug administration were immediately centrifuged at 3000 g for 15 min at 4°C, plasma was separated and stored in vials containing 10 µl of ortho phosphoric acid per ml plasma at -20°C until analysed. After a wash-out period of 7 days, the study was repeated in the same manner to complete the crossover design. The order of receiving the test and reference product for each subject during the two periods of the study was determined according to a randomization scheme. The plasma samples obtained at various time intervals were analysed by validated HPLC method.

Pharmacokinetic analysis

The plasma concentration profile obtained for active metabolites (i.e. T & TG) was fed into S-inverse (S-inv), computer software on BASICA® Version 1.12, to determine the pharmacokinetic parameters. The maximum metabolites (i.e. T & TG) concentrations (C_{max}) and the corresponding peak time (T_{max}) were determined by checking the individual drug plasma concentration-time profiles. The elimination rate constant (K_{el}) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration-time profile. The elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve extrapolated to infinity (AUC_{0-inf}) was calculated as $AUC_{0-t} + C_t / K_{el}$, where C_t is the last measurable concentration.

Statistical analysis

For the purpose of bioequivalence analysis AUC_{0-t} , AUC_{0-inf} and C_{max} of active metabolites (i.e. T and TG) were considered as primary variables. Bioequivalence of two formulations was assessed by means of an analysis of variance (ANOVA) for crossover design and calculating 90% confidence interval of the ratio of test/reference using log transformed data on a Microsoft excel®. The formulation was considered bioequivalent when the difference between two compared parameters was found statistically insignificant ($p > 0.05$) and confidence interval for these parameters fell within 80-125%.

Results

Under the chromatographic conditions employed the sample showed good separation of AG, T, TG and internal standard from the endogenous impurities of plasma. The Retention time of the AG, T, TG and IS was found to be 8.20 ± 0.2 , 5.3 ± 0.2 , 4.0 ± 0.2 and 4.9 ± 0.2 mins, respectively (Figure 2). The results of validation parameters were as follows:

The peaks of AG, T, TG and IS were well resolved. No endogenous peak from plasma was found to interfere with the elution

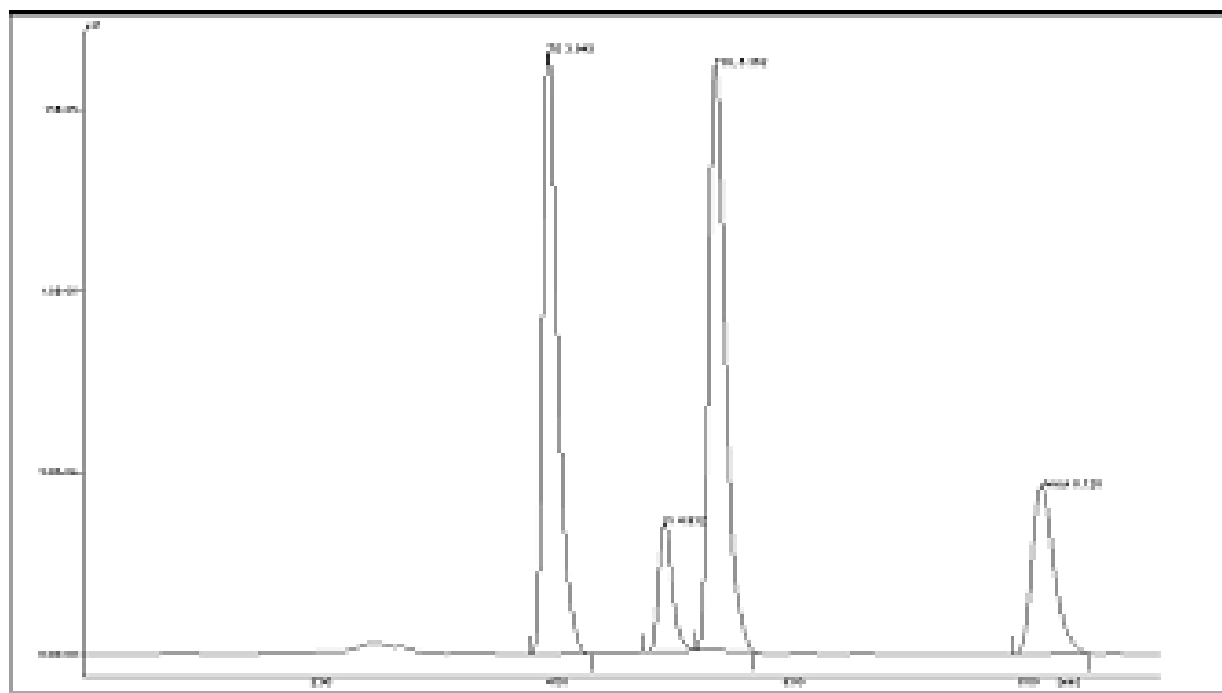


Figure 2: Representative chromatogram of calibrator solution of AG, T, TG and IS. Representative chromatogram of standard solution, showing peaks of tolmetin glycinamide (TG) at Rt-4.012 min, internal standard (IS) at Rt-4.877 min, tolmetin (Tol) at Rt-5.402 min and amtolmetin gauacil (Amtol) at Rt-8.1258 min.

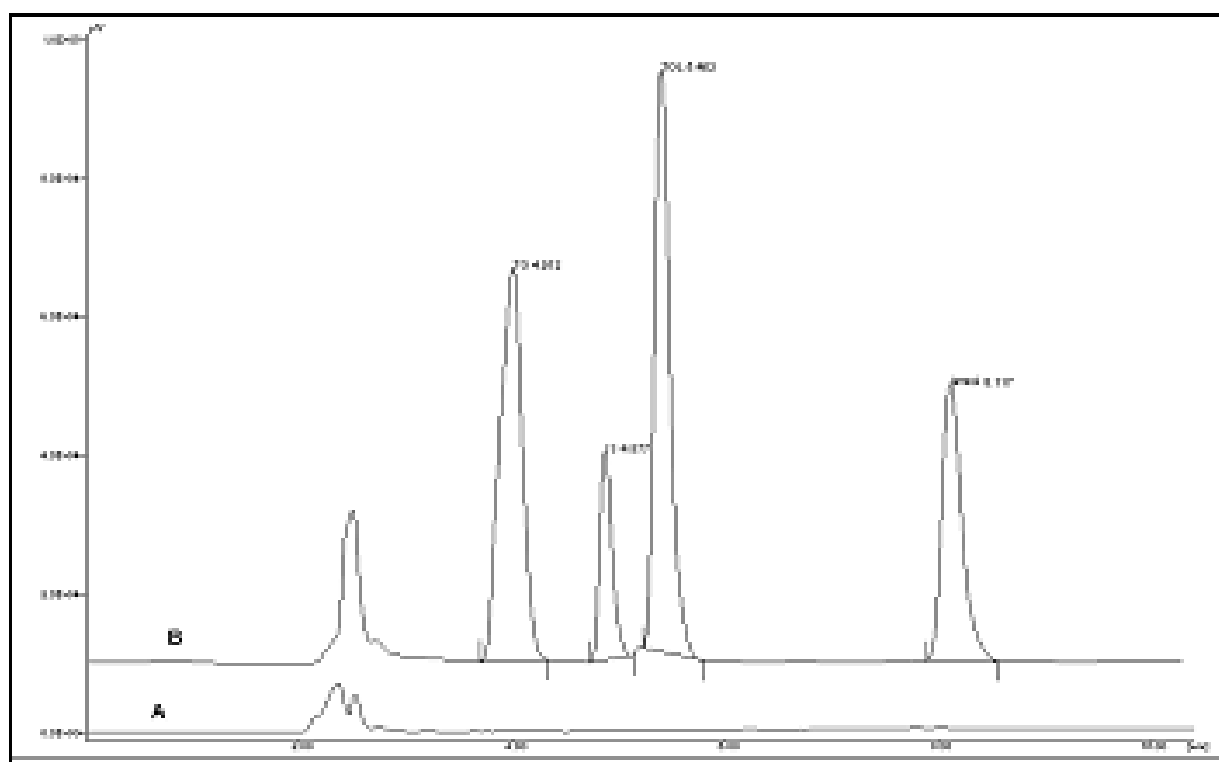


Figure 3: Comparative HPLC Chromatograms. Selectivity representative chromatograms (A) blank plasma chromatogram and (B) Plasma spiked with tolmetin glycinamide (TG) at Rt-3.943 min, internal standard (IS) at Rt-4.932 min, tolmetin (Tol) at Rt-5.362 min and amtolmetin gauacil (Amtol) at Rt-8.117 min.

of AG, T, TG or the IS. Selectivity of the method was thus proven by the absence of interfering peaks near the Rt of AG, T, TG and IS. Representative chromatograms of human blank plasma and human blank plasma spiked with AG, T, TG and IS are shown in Figure 3(A-B), respectively.

The calibration curve of AG, T and TG was linear over the

working concentration range of 0.25 – 20 µg/ml in human plasma with seven-point calibration with accurate r^2 values. The LOQ of AG, T and TG in plasma was verified as 0.25 µg/ml, as this was the lowest concentration assessed at which the accuracy was between 80 and 120% and precision was within 20 %. The LOD was 0.1 µg/ml at a signal-to-noise ratio of 3.

The results for accuracy and precision for AG, T and TG quality control standards are presented in Table 1. All the values of accuracy and precision including LOQ fell within the limits considered as acceptable. The recovery of AG, T, TG and IS from 1 ml of plasma was measured for the quality control standards are tabulated in Table 2, expressed as a percentage, obtained for AG, T, TG and IS.

Three freeze-thaw cycles of the quality control standards appeared to have no effect on stability of the analyte. Quality control samples stored in a freezer at -20°C remained stable for at least one month. These studies suggested that human plasma samples containing AG, T and TG can be handled under normal laboratory conditions without significant loss of compound (Table 3a-c).

Concentration (µg/ml)		TG	T	AG
Intra Day (n=6)				
0.25	Mean ± SD	0.240 ± 0.022	0.247 ± 0.024	0.241 ± 0.023
	%CV	9.194	9.735	9.554
	% Accuracy	95.933 ± 8.820	98.677 ± 9.606	96.267 ± 9.197
2.5	Mean ± SD	2.444 ± 0.180	2.409 ± 0.160	2.505 ± 0.195
	%CV	7.346	6.657	7.768
	% Accuracy	97.740 ± 7.180	96.367 ± 6.415	100.2 ± 7.783
20	Mean ± SD	19.892 ± 0.146	19.896 ± 0.223	19.880 ± 0.125
	%CV	0.736	1.123	0.627
	% Accuracy	99.458 ± 0.732	99.481 ± 1.117	99.398 ± 0.623
Inter Day (n=6)				
0.25	Mean ± SD	0.260 ± 0.017	0.255 ± 0.020	0.254 ± 0.021
	%CV	6.718	7.921	8.189
	% Accuracy	104.133 ± 6.718	102.00 ± 8.080	101.467 ± 8.309
2.5	Mean ± SD	2.467 ± 0.135	2.472 ± 0.175	2.522 ± 0.132
	%CV	5.488	7.097	5.235
	% Accuracy	98.680 ± 5.416	98.880 ± 7.017	100.867 ± 5.280
20	Mean ± SD	19.967 ± 0.381	20.095 ± 0.408	20.077 ± 0.443
	%CV	1.906	2.031	2.208
	% Accuracy	99.833 ± 1.903	100.476 ± 2.040	100.384 ± 2.216

Table 1: Accuracy and precision of the method for determining the concentration of AG, T, TG in plasma samples (n=6).

Concentration (µg/ml)		TG	T	AG
0.25	% recovery (Mean ± SD) (n=3)	93.92 ± 2.367	94.68 ± 2.987	92.23 ± 1.602
	%CV	2.520	3.154	1.737
2.5	% recovery (Mean ± SD) (n=3)	93.227 ± 3.732	92.853 ± 2.751	90.819 ± 5.358
	%CV	4.003	2.963	5.900
20	% recovery (Mean ± SD) (n=3)	93.100 ± 3.126	91.110 ± 1.671	90.893 ± 1.914
	%CV	3.357	1.834	2.106
Internal Standard				
% recovery (Mean ± SD) (n=3)		90.153 ± 1.296		
%CV		1.438		

Table 2: Recoveries of AG, T, TG and IS from Drug Free Human Plasma.

Number of days stored	0.25 µg/ml		2.5 µg/ml		20 µg/ml	
	Mean ± SD (n = 3)	% C.V.	Mean ± SD (n = 3)	% C.V.	Mean ± SD (n = 3)	% C.V.
3-Freeze thaw studies	0.250±0.01	4.000	2.523±0.02	0.605	20.287±0.11	0.532
5 days	0.240±0.01	4.167	2.427±0.07	2.805	20.160±0.15	0.753
15 days	0.233±0.02	6.547	2.343±0.06	2.427	19.897±0.06	0.286
30 days	0.223±0.01	5.170	2.247±0.07	3.126	19.683±0.04	0.178

Table 3a: Stability studies. Stability of tolmetin glycinamide in human plasma.

Number of days stored	0.25 µg/ml		2.5 µg/ml		20 µg/ml	
	Mean ± SD (n = 3)	% C.V.	Mean ± SD (n = 3)	% C.V.	Mean ± SD (n = 3)	% C.V.
3-Freeze thaw studies	0.247±0.006	2.341	2.493±0.042	1.670	20.280±0.061	0.300
5 days	0.237±0.006	2.440	2.413±0.055	2.282	19.770±0.123	0.622
15 days	0.227±0.015	6.739	2.327±0.057	2.444	19.607±0.155	0.792
30 days	0.220±0.01	4.545	2.217±0.05	2.271	19.397±0.157	0.809

Table 3b: Stability studies. Stability of tolmetin in human plasma.

Number of days stored	0.25 µg/ml		2.5 µg/ml		20 µg/ml	
	Mean ± SD (n = 3)	% C.V.	Mean ± SD (n = 3)	% C.V.	Mean ± SD (n = 3)	% C.V.
3-Freeze thaw studies	0.247±0.012	4.681	2.540±0.060	2.362	20.173±0.068	0.337
5 days	0.250±0.01	4.000	2.547±0.07	2.758	20.160±0.066	0.325
15 days	0.230±0.01	4.348	2.407±0.081	3.359	19.800±0.072	0.364
30 days	0.220±0.01	4.545	2.257±0.068	3.019	19.640±0.053	0.269

Table 3c: Stability studies. Stability of amtolmetin guacil in human plasma.

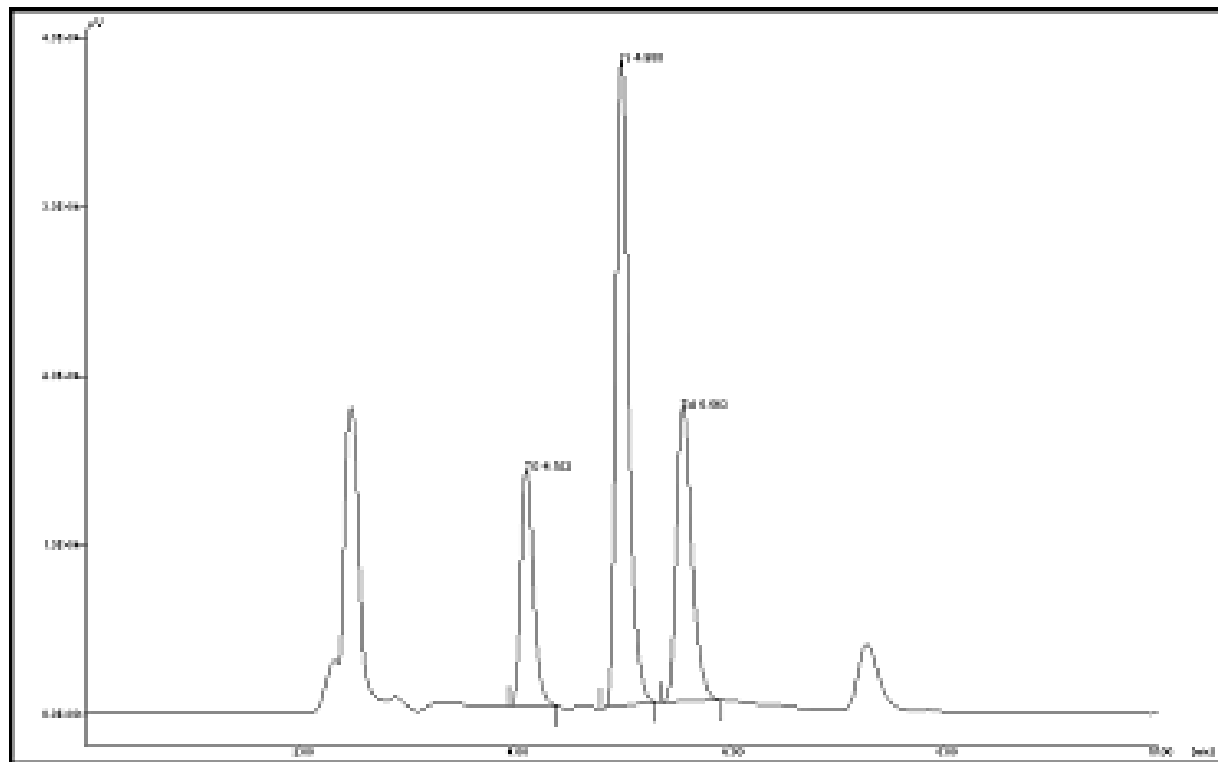


Figure 4: Representative chromatogram of volunteer plasma sample. Representative Chromatogram of Volunteer Plasma Sample Collected at 10 min after Dosing with 600 mg of Reference AG Tablet - peaks of tolmetin glycinamide (TG, Rt-4.102 min), internal standard (IS, Rt-4.988 min), tolmetin (Tol, Rt-5.563 min).

Parameters	Test	Reference	P value ^ψ	90 % CI ^φ (80-125 %)	T/R ratio
C _{max} (µg/ml)	19.527±0.809	19.291±0.855	0.35	99.65-101.23	101.3088
T _{max} (h)	1.08±0.056	1.17±0.077	0.31		
K _{elc} (l/hr)	0.371±0.063	0.352±0.052	0.93		
T _{1/2} (h)	2.994±0.478	2.520±0.414	0.34		
AUC _{0-t} (µg.h/ml)	90.024±2.645	90.818±3.173	0.89	100.31-104.24	99.30043
AUC _{0-inf} (µg.h/ml)	94.671±3.904	98.117±5.573	0.69	100.25-105.94	97.16596

Values are given as standard error of mean

(^ψNon-significant difference at 95 % confidence limits)

(^φStatistics were applied on log-transformed data, n= 12)

Table 4a: Pharmacokinetic Parameters after Single Oral Dose of 600 mg Amtolmetin Guacil Tablet in 12 Healthy Male Volunteers. Pharmacokinetic Parameters of Tolmetin.

Parameters	Test	Reference	P value ^ψ	90 % CI ^φ (80-125 %)	T/R ratio
C _{max} (µg/ml)	10.058±0.318	10.398±0.258	0.21	98.72-102.16	96.47
T _{max} (h)	1.42±0.164	1.38±0.065	0.89		
K _{elc} (l/hr)	0.274±0.028	0.255±0.028	0.50		
T _{1/2} (h)	2.862±0.311	3.093±0.318	0.50		
AUC _{0-t} (µg.h/ml)	49.09±2.63	50.603±2.725	0.44	101.23-103.33	97.15
AUC _{0-inf} (µg.h/ml)	50.925±2.77	53.584±2.94	0.20	101.18-104.64	95.16

Values are given as standard error of mean

(^ψNon-significant difference at 95 % confidence limits)

(^φStatistics were applied on log-transformed data, n= 12)

Table 4b: Pharmacokinetic Parameters after Single Oral Dose of 600 mg Amtolmetin Guacil Tablet in 12 Healthy Male Volunteers. Pharmacokinetic Parameters of Tolmetin Glycinamide.

Application to bioequivalence study

The validated method has been used successfully applied to quantify the concentration of T and TG in human plasma samples after the administration of a single oral dose of amtolmetin (600 mg). The sampling duration was 24 h, however after 12 h; T and TG was not detectable in the plasma sample by UV detector. Figure 4 shows the chromatograms of plasma samples obtained from a volunteer at 10 min after dosing. The observed pharmacokinetic parameters, ratio of test/reference (T/R), 90% percent confidence intervals (90 CIs) and their statistical values for the active metabolites (i.e. T and TG) after an oral administration of 600 mg amtolmetin guacil tablets were within the range of 80-125% in accordance with the Food and Drug Administration Bioequivalence Guideline [7] and are presented in Table 4(a-b). There were no remarkable differences between test formulation and reference formulation of amtolmetin guacil.

Discussion

The present work describes a new, simple, sensitive and rapid isocratic method to quantify amtolmetin and its active metabolites from human plasma using HPLC with UV detection and its application to *in vivo* single dose pharmacokinetic study of test and standard formulation.

To develop suitable chromatographic conditions, initially pure sample of AG, TG and T was used and the standard solutions were prepared in acetonitrile. Preliminary separation of AG and its metabolites (TG and T) were tried on a C18 column. Chromatograms of two different concentrations of AG and its active metabolites (TG and T) were studied using HiQsil column. On C18 column the peaks of active metabolites were not well resolved in varying proportion of methanol, acetonitrile and acetic acid at different pH levels tested. Hence, chromatographic separation was tried on C8 column. Various proportions of acetonitrile, methanol with acetic acid (0.1% - 1%) were tried for separation of amtolmetin and its metabolites. It was found that a mixture of acetonitrile:methanol:1% acetic acid in the ratio of 55:5:40 (v/v) delivered at a flow rate of 1 ml/min with detection at 313 nm could achieve our purpose and was finally adopted. Peak shape and resolution of AG and its metabolite were better with C8 column with the mobile phase containing acetonitrile:methanol:1% acetic acid in the ratio of 55:5:40 (v/v) delivered at a flow rate of 1 ml/min.

For internal standard, drugs that have similar solubility properties as AG, T and TG and can be resolved from the analyte and other plasma impurities were chosen during method development. Drugs like dexibuprofen, torsemide, trimebutine and coumarin were attempted for selection as internal standard. The drugs except coumarin tried were found to be either overlapping with retention time of metabolite (torsemide) or eluting with plasma impurities (trimebutine) or the retention time was too high (dexibuprofen) under the optimized chromatographic conditions. AG, TG, T and coumarin (IS) showed good resolution with retention times of 8.20 ± 0.2 , 5.3 ± 0.2 , 4.0 ± 0.2 and 4.9 ± 0.2 mins, respectively, under optimized chromatographic conditions. Hence, coumarin was selected as an internal standard.

Detection of a drug and its metabolite in biological media is usually complicated by the matrix. Because of this, various types

of cleanup steps such as solvent extraction and chromatographic method are employed to effectively separate drug components from endogenous impurities. The ultimate sensitivity and selectivity of the method may be limited to the cleanup procedures. To avoid loss due to cleanup procedure and to increase recovery of analyte of interest the number of cleanup steps in a sample preparation should be kept to minimum (Smith and Stewart, 1991). The sample preparation of AG and its active metabolites was based on simple protein precipitation technique. Amtolmetin and its active metabolites are freely soluble in solvents like acetonitrile and methanol. Thus initially protein precipitation method was attempted with solvents like methanol and acetonitrile. The method was not selective when protein precipitation was done with methanol, but precipitation with 1.5 ml of acetonitrile gave cleaner chromatograms along with good recoveries of drug, metabolites and internal standard.

Once the method was developed, validation of the method was done for its selectivity, limit of detection and quantitation, *intra-day* and *inter-day* accuracy and precision, linearity, recovery and stability in plasma as per USFDA guidelines. The validation parameters of the method were found to be well within the guideline set limits. The utility of the developed analytical method for the determination of AG, its metabolites and IS was demonstrated in the *in vivo* single dose pharmacokinetic study of AG tablets, wherein the method was successfully employed to determine the TG and T concentrations in human plasma samples.

The method developed and validated for the quantification of AG and its active metabolites (T and TG) was applied to bioequivalence study of AG 600 mg tablet (test and standard formulations), wherein the method was successfully applied to compare the plasma concentration of T and TG from healthy human volunteers plasma samples after administration of single dose of 600 mg AG test and reference tablets. The study was carried out in 12 Indian healthy male volunteers after a single oral dose of 600 mg amtolmetin guacil (Test and Reference) tablet. Various pharmacokinetic parameters for active metabolites of AG (i.e. T and TG) were compared between test and reference formulation. Both the formulations were well tolerated by all the volunteers in both the periods of the study. No clinical adverse events occurred during the study. The pharmacokinetic study was performed upto 24 h, however after 12 h; T and TG was not detectable in the plasma sample by UV detector. The half-life of both the active metabolites from the study was about 3-4 h. For comparing the bioequivalence of two products, data of 3 half-life for active drug is sufficient and which is about 12 h. Hence, the developed method is sensitive enough to apply for bioequivalence study of 600 mg AG tablets.

This paper describes a simple, rapid and sensitive method for the determination of amtolmetin and its active metabolites in human plasma using high-performance liquid chromatography with ultraviolet detection. The sensitivity and simplicity of the method makes it suitable for pharmacokinetic studies and was successfully applied to a bioequivalence study of amtolmetin guacil 600 mg tablet in Indian healthy male volunteers. The statistical comparison of AUCs and C_{max} clearly indicated no significant difference in the two formulations of 600 mg of amtolmetin guacil

tablets. 90% confidence interval for the AUC_{0-t} , AUC_{0-inf} and C_{max} indicates that the values were entirely within the bioequivalence acceptance range of 80-125 % (using log transformed data). Based on these results it was concluded that formulation 'Test' is bioequivalent with formulation 'Reference' and can be interchangeable in clinical practice.

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