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Ion-pair HPLC Method for the Quantification of Metformin in Human Plasma and Its Application to a Pharmacokinetic Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ET, LD and GB conceived and designed the experiment. Author ET performed the experiment and collected the data. Author LD gave technical support and conceptual advice. Authors ET and LD designed and implemented the model. Authors ET and LD managed the analyses of the study. During a research project the involved scientists discuss freely and influence each other. Author ET wrote the protocol and the manuscript. Author GB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To develop and validate a rapid, selective and sensitive ion-pairing HPLC-UV method for the determination of metformin in human plasma, using a conventional reverse phase column.
Study Design: Experimental study.
Place and Duration of Study: Department of Department of Biomedical Sciences, Faculty of Medicine, University of Medicine, "Mother Tereza" hospital center, between November 2014 and February 2015.
Methodology: Ion-pair separation followed by UV detection performed on deproteinised and dichloromethane washed plasma samples was chosen for the determination of metformin. The separation was performed on an analytical LiChrocart® 100 RP 18 (125 × 4.0 mm i.d., 5 µm particle size) C18 column. A mobile phase consisting of acetonitrile and 10 mM sodium phosphate buffer

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(pH=6.0; 32.5:67.5, v/v) and sodium dodecyl sulfate (0.3%) was pumped at an isocratic flow rate of 1.25 mL/minute, and quantification was achieved at 236 nm using a UV/Vis DAD.
Results: The calibration curves were linear (r > 0.9998) in the concentration ranges of 50-1600 ng/mL for metformin in plasma. The assay enables the measurement of metformin for therapeutic drug monitoring with a minimum detectable limit of 18 ng/ml. The coefficients of variation for inter-day and intra-day assay were within the range of clinical usefulness. Absolute recovery was found to be > 90% for all three concentrations of plasma quality controls studied.
Conclusion: The proposed method was found to be rapid, precise and accurate for quantification of metformin in human plasma. This method was successfully applied to a pharmacokinetic study at humans through oral administration.

Keywords: Metformin; ion-pair HPLC method; human plasma; quantification; pharmacokinetics.

ABBREVIATIONS

AUC0–∞: Area Under the curve extrapolated to infinity; AUC0–T: Area under the curve up to the last sampling time; CMAX: The Maximum plasma concentration; TMAX: The time to reach peak concentration; T1/2: The elimination half-life; KEL: The apparent elimination rate constant.

1. INTRODUCTION

Metformin hydrochloride has been successfully used for many years in the treatment of type 2 diabetes. Metformin is primarily absorbed from the small intestine. The extent of metformin absorption is improved when the gastrointestinal motility is slowed [1]. Following absorption plasma protein binding is negligible, and it is excreted unchanged in the urine [2]. For pharmacokinetic studies, a sensitive method that allows an accurate measurement of low concentrations of metformin in plasma is required.

Metformin is a small highly polar molecule (pKa =2.8, 11.5, logP octanol: water = -2.6) which has great solubility in water and poor solubility in lipids so it is very difficult to extract it from the aqueous plasma matrix [3]. There are many methods reported to analyze metformin hydrochloride in plasma by HPLC (Table 1). In clinical laboratories four important factors must be considered for the plasma metformin assay to be functional: an appropriate LLOQ, simple and efficient sample pre-processing, reasonable elution time and low cost.

Previously described methods suffered from several disadvantages, such as lack of sensitivity [4-10], using complex extraction procedures which are difficult, costly and time consuming [11-16], required the use of ultrafiltration and a column-switching system [8], required the gradient mobile phases [17,6], required relatively large sample volume [17,16]. Some dilute the sample excessively and require nitrogen drying [17,13,14]. Most of these reported methods are precise and accurate, however employ such specific columns [18,12,19,15] which are not normally available in common laboratories. In view of this, a simple reverse phase HPLC method has been developed and validated to accurately and precisely estimate metformin in human plasma using commonly available C_{18} column, ultraviolet detector, and acetonitrile for fundamental processes such as deproteinization of plasma and extraction of metformin. The intent of this application was to develop an ion-pair HPLC method for the analysis of metformin in plasma using a conventional reverse phase column.

This paper describes a rapid and sensitive HPLC method, which enables the determination of metformin with good accuracy at low drug concentrations in plasma using only a one step protein precipitation and dichloromethane wash method. With a small volume plasma sample, this method was found to reach the levels of selectivity and reproducibility required for pharmacokinetic study in healthy Albanian volunteers.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Metformin HCl (purity: 100%) was a gift from Profarma Sh.A. (Tirana, Albania). Metformin film-coating tablet (850 mg metformin HCl) was obtained by pharmacies (Albania).

HPLC-grade acetonitrile (Merck, Darmstadt, Germany), sodium dodecyl ulphate (SDS) (97% analytical grade) (BASF SE, Ludwigshafen, Germany), and ortho-phosphoric acid (85% analytical grade) (Prolabo, Briare, France) were used in the study. For mobile phase filtration, Nylon membrane filters were used (type: hydrophilic Nylon membrane filter) (EMD Millipore) (47 mm, 0.45 µm pores). Water used for the analysis was prepared via a Direct Q-UV-Ultrapure® water system procured from EMD Millipore. Blank human plasma was obtained from healthy volunteers according to Good Clinical Laboratory Practice (GCLP). Blood was collected in heparinized VacuCyte tubes (Zhejiang U-REAL Medical Technology, Co., LTD, Zhe Jiang, China).

2.2 Instrumentation and Chromatographic Conditions

To prepare samples, the following equipment was used: A 55702 centrifuge (Bioblock Scientific, Illkirch, France); an analytical balance (Denver Instrument, Bohemia, NY, USA); an Ultrasonic cleaning bath 1210 (Branson Ultrasonics, Danbury, CT, USA); a vortex mixer (VELP Scientifica, Usmate, Italy); and pH- meter (Denver instrument).

The chromatographic study was carried out using an Agilent 1200 HPLC system (Agilent Technologies), which featured a built-in binary pump, manual injector (Rheodyne, 20 µL loop), and an ultraviolet (UV)/visible diode array detector (Agilent 1200; Agilent Technologies). The chromatograms were recorded using the Chromatographic Work Station software (ChemStation for LC3D system Rev.B04.02[118]), which was run on a computer system used for data collection and processing. The separation was performed on an LiChroCart® 100 RP 18 (125/× 4.0 mm i.d. 5 µm, particle size) column (ISS, Surrey, UK). The mobile phase was prepared by mixing 0.01 M of sodium phosphate buffer (pH=6.0), 0.3% SDS, and acetonitrile in a ratio of 67.5:32.5, adjusting with H_3PO_4 to 6.0 as necessary. The mobile phase was prepared daily, filtered through a 0.45 µm porosity Nylon filter membrane, and ultrasonicated for 30 minutes before use. The flow rate and the column temperature were 1.25 mL/minute and 50℃, respectively. The detection of metformin was carried out at 236 nm. Under these conditions, the retention time of metformin was 4 minutes.

2.3 Preparation of Stock, Standard Solutions and Quality Control Samples

Stock solution (500 µg/mL) and appropriate dilutions of metformin were prepared in water and stored at /4℃-8℃. No change in stability over the period of 4 weeks was observed. The working standards, obtained by diluting the stock solution in drug-free plasma, were routinely prepared to contain 50-1600 ng of metformin per mL. Three concentrations (quality control) were chosen from the high, medium and low range of the standard curve (300, 800 and 1500 ng/ml). These solutions were freshly prepared for each analytical run.

2.4 Plasma Sample Preparation

To 500 µl plasma were added acetonitrile (1:1) and the mixture was vortexed for 30 seconds at 25 Hertz. The mixture was centrifuged at 10000 rpm for 10 minutes. The upper layer (about 0.75 mL) was separated and mixed with 1.5 mL dichloromethane for 30 seconds and centrifugated at 5000 rpm for 10 minutes. Then 20 µL of supernatant was injected into liquid chromatograph.

2.5 Method Validation

The method was validated for selectivity, linearity, limit of quantification, accuracy, precision and recovery as per the international guidelines [20].

2.6 Application to Pharmacokinetic Study of Metformin in Human Plasma

The validated method has been successfully used to quantify metformin concentrations in human plasma. The study was conducted according to current Good Clinical Practice guidelines and approved by an authorized National Medical Ethics Committee. Metformin was administered in a single dose of 850 mg to healthy volunteers after over night fasting. There was a total of 14 blood collection time points. The blood samples were collected in separate heparinized VacuCyte tubes. The plasma from these samples was separated by centrifugation at 3500 rpm for 10 minutes. The plasma samples thus obtained were stored at –20℃ until analysis. After analysis the pharmacokinetic parameters were computed using software R with package “bear”, version 2.6.5 (Kaohsiung, Taiwan).

3. RESULTS AND DISCUSSION

3.1 Method Development

Several methods for the measurement of plasma metformin have been developed, most of which have been based on HPLC with spectrophotometric detection in the range of 230–240 nm (Table 1). Metformin can be eluted using silica [12,19], cyano [7], C18 [4,11,17,5,13,14,21,16,9], phenyl [18,6,15] or cation-exchange columns [22,8,10] with mobile phases consist of a mixture of phosphate buffer (pH 2.4–7.5) and acetonitrile. Sample pre-treatment methods include protein precipitation [4,17,18,5,6,21,19,7,9,10], solid phase extraction [13-15], metformin derivatization [23,24], ultrafiltration [8] and liquid–liquid extraction followed by back extraction [12,16]. Due to the high polarity of metformin, which makes it very difficult to extract it from biological fluids using organic solvents, protein precipitation is the most common method used for plasma sample preparation. It has been commented that the use of this simple and rapid procedure sacrifices sensitivity and is not effective in removing endogenous substances [12,14,8]. In order to overcome these problems, David et al. [23] and Tache et al. [24] describe methods based on derivatization reactions; Gabr et al. [11]; Amini et al. [12] and Keal and Somogyi [16] describe liquid–liquid extraction procedures followed by back extraction; AbuRuz et al. [13,14] describe ion pair SPE procedure but these are too complicated and time-consuming for application in bioequivalence assays. In the present study, protein precipitation with acetonitrile was found to be highly effective in removing endogenous substances, since all of such components had retention times lower than 4 min and did not interfere with metformin (Fig. 1). The result of these experiments led to the conclusion that the recovery of metformin from plasma increased by dichloromethane wash step. The best washing quantity (at the washing step) was found to be 1.5 mL dichloromethane. It also produced higher average recoveries for the analyte than previously described method which used protein precipitation and dichloromethane wash [18], liquid–liquid extraction [11], liquid–liquid extraction followed by back extraction [16], or solid phase extraction [15] for sample preparation. This method has an elution time of 7 min per plasma sample that is shorter than the method with similar sensitivity [17]. Also the retention time and the run time is shorter than procedure described by AbuRuz et al. [13,14]. Peak height was more sensitive than peak area. Critical pair (impurity) was too close to main peak to not get baseline to baseline integration in peak area in the standard. Also, the resolution requirement was fulfill with peak height.

3.2 Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Selectivity of the method was evaluated by analyzing six different blank plasma samples to investigate the potential interferences at the HPLC retention time for the analyte. The selectivity of the method was tested by comparing the chromatograms of blank plasma and the spiked plasma. Under the above conditions, the retention time of metformin was 4.1 minutes. As shown in Fig. 1, there was no interference at the retention time of metformin.

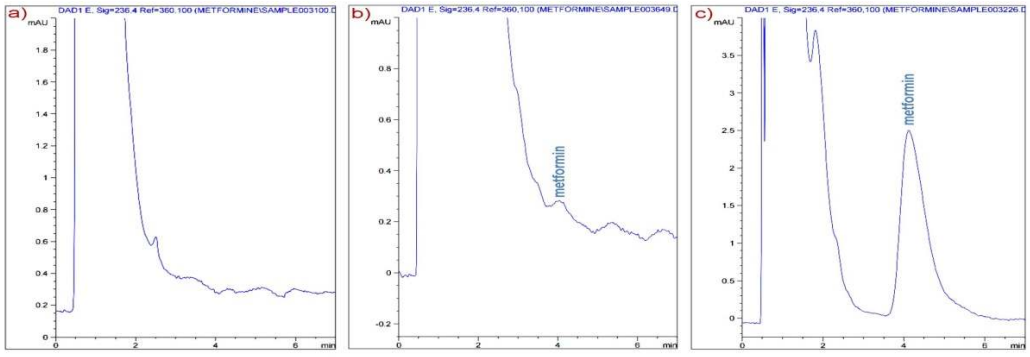

Fig. 1. Chromatograms of (a) blank plasma; (b) blank plasma spiked with 50 ng/mL metformin; (c) plasma sample from a healthy volunteer 1 hour after oral administration of an 850 mg of metformin tablet

Table 1. Comparisons of some published methods for assaying metformin in human plasma

Validated plasma LOQ (ng/mL)	Sample preparation	Analytical column	Retention time (min)	Run time (min)	Reference
50	Protein ppt and DCM wash	RP-C18	4.1	7	Current method
125	One step protein ppt	C18	9.932	15	Chhetri et al. 2014 [4]
7.8	LLE	C18	9.5	16	Gabr et al. 2010 [11]
50	Protein ppt with ACN and evaporation to dryness	C18	9.375	20	Ranetti et al. 2009 [17]
30	Protein ppt and DCM wash	phenyl	7.5	10	Porta et al. 2008 [18]
330	Protein ppt	C18	4.74	8	Wanjari et al. 2008 [5]
250	Protein ppt step with ACN	Phenyl	5.26	10	Yardimci et al. 2007 [6]
15.6	LLE	Silica column	5.7	10	Amini et al. 2005 [12]
Method 1 (5) Method 2 (16.5)	Ion pair SPE	C18	4.7	20	AbuRuz et al. 2005 [13]
5	Ion pair SPE	C18	4.85	15	AbuRuz et al. 2003 [14]
Not specified	One step protein ppt	C18	3.4	6	Zarghi et al. 2002 [21]
10	Protein ppt and DCM wash	Silica column	7.8	10	Cheng and Chou 2001 [19]
60	Deproteination using perchloric acid	Cyano	5.9	8	Yuen and Peh 1998 [7]
100	Ultrafiltration	CX	5	12	Vesterqvist et al. 1998 [8]
50	SPE C8	Phenyl	2.8	6	Huupponen et al. 1992 [15]
10	IPE	C18	3.3	9	Keal and Somogyi 1986 [16]
75	Protein ppt with ACN and evaporation	C18	8	10	Benzi et al. 1986 [9]
200	TCA/ Protein ppt	CX	8	12	Charles et al. 1981 [10]

Abbreviations: LOQ=lower limit of quantitation, SPE=solid phase extraction, protein ppt= protein precipitation, ACN= acetonitrile, DCM wash=dichloromethane wash, IPE= ion pair extraction, LLE= liquid-liquid extraction, TCA= trichloroacetic acid, CX= cation exchange

3.3 Linearity

The linearity was determined from the standard calibration curve. Six non-zero samples (50, 200, 300, 400, 800, and 1600 ng/ml) including the lower limit of quantification (LLOQ i.e. 50 ng/ml) were used to draw the standard calibration curve (n= 3). The calibration curve was obtained by plotting chromatographic peak height ratio versus concentration of metformin hydrochloride. Samples were prepared and injected same day. The calibration curve (see Fig. 2) was linear in the given range (R^2 = 0.9991).

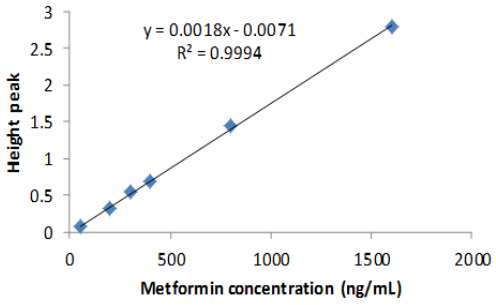

Fig. 2. Calibration curve for metformin (50-1600 ng/mL) in human plasma

3.4 Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOD and LOQ were determined separately on the basis of a standard calibration curve. The residual standard deviation of the regression line or the standard deviation of the y-intercepts of the regression lines were used to calculate the LOD and LOQ. The sensitivity of the proposed method was estimated in terms of LOD and LOQ; LOD=3.3 SD/S and LOQ=10 SD/S, where SD is the residual standard deviation of the regression line and S is the slope of the line. The LOQ and LOD as per these criteria were found to be 50 ng/mL and 18 ng/mL, respectively.

So, this method was found to be sensitive enough to apply for pharmacokinetic studies.

3.5 Precision and Accuracy

The intra-day assay precision and accuracy were obtained by analyzing six replicates of QC samples on a single day. The inter-day assay precision and accuracy were obtained by analyzing six replicates of QC samples on 3 different days. Intra-day and inter-day precisions of the method were expressed by [standard deviation/mean concentration] ×100%. Accuracy of the method was expressed by [(mean measured concentration–nominal concentration)/ nominal concentration] × 100%. The relative percentage error (RE%) of the mean value should be within ±15% at each concentration. The precision was required to be less than 15% at each concentrations. As shown in Table 2, the values for both intra- and inter-day accuracy and precision were found to be within the acceptable criteria.

3.6 Recovery

The extraction recovery for the analyte for three levels of QC samples was assessed by comparing the peak height for extracted spiked plasma samples with the peak area for pure compounds of the same concentrations in solvent. This method gave a good recovery (Table 3).

3.7 Stability

The stability tests of the analytes were designed to cover expected conditions concerning the handling of clinical samples. Metformin stability in processed and unprocessed plasma samples was investigated. Metformin in processed samples (300 and 800 ng /ml) was found to be stable for 24 hours at room temperature and 48 hours at -20ºC. Metformin in unprocessed plasma samples was stable for at least 24 hours at room temperature, 30 days at -20ºC, or after three freeze-and thaw cycles. Data are summarized in Table 4. Metformin in stock solutions (500 µg/ml in HPLC grade water) were found to be stable for 48 hours at room temperature and at least 4 weeks at 2° - 8℃. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (≤ 15% RE) and precision (≤ 15% RSD). The results indicated that metformin were stable for the entire period of the experiment.

3.8 Application to Pharmacokinetic Study of Metformin in Human Plasma

The validated method has been successfully used to quantify metformin concentrations in human plasma. The aim of our study was to develop a rapid and sensitive method for the routine analysis of biological samples in pharmacokinetic metformin research. This method is well suited for routine application in the clinical laboratory because of the simple extraction procedure and good sensitivity. Over 520 plasma samples were analyzed by this method. In this study plasma concentrations were determined in twenty healthy volunteers, who received 850 mg of metformin each. Plasma drug concentration–time data were subjected to noncompartmental pharmacokinetic analysis using linear trapezoidal rule.

Table 2. Intra-day and inter-day precision and accuracy for the detection of metformin HCl in human plasma

Concentration added (ng/mL)	Intra-day (n=6)			Inter-day (n=21)		
	Concentration found (ng/mL)			Concentration found (ng/mL)		
	(mean±SD)	Precision (RSD, %)	Accuracy (RE, %)	(mean±SD)	Precision (RSD, %)	Accuracy (RE, %)
300	312.2±13.09	4.2	4.1	304±13.78	4.5	1.36
800	819.7±23.06	2.8	2.5	791.8±38.7	4.9	1.02
1500	1591.8±23.06	1.4	6.1	1547.92±51.97	3.4	3.19

RSD-relative standard deviation; RE-Relative error

Table 3. Recovery of metformin hydrochloride at three concentrations (n = 6)

Concentration added (ng/ml) (n=6)	Mean recovery % ± SD	RSD %
300	95.3±0.92	0.97
800	98±0.6	0.62
1500	100.7±0.6	0.59

Bioequivalence studies protocols generally recommend plasma sample collection for a time period corresponding to three to four times the drug plasma elimination half-life [25]. Fig. 3 shows the mean plasma concentration-time curve of metformin after oral administration of 850 mg to 20 healthy volunteers: Plasma concentration reached a maximum 2.325±0.654 h after dosing with a level of 1771.750±503.526 ng/ml. The pharmacokinetic parameters such as C_{max}, T_{max}, $t_{1/2}$, k_{el}, AUC_{0-t}, $AUC_{0-\infty}$ for metformin are summarized in Table 5. The half-life is somewhat longer, the apparent elimination rate constant (K_{el}) is lower and the C_{max} higher than previously observed with standard 850 mg tablets of metformin hydrochloride [26]. The time to reach peak concentration (T_{max}) value was in close proximity when compared with earlier reported value [26]. For metformin, mean peak plasma concentration of about 1771 ng/ml (Fig. 3) will produce plasma concentrations after a time period corresponding to four metformin half-lives of approximately 100 ng/ml. Since the method LOQ was 50 ng/ml, its sensitivity is adequate for bioavailability studies. Area under plasmatic concentration time curve from 0 to 14 h (AUC_{0-14h}) and from 0 h extrapolated to infinity ($AUC_{0-\infty}$) were calculated and the ratio $AUC_{0-14h}/AUC_{0-\infty}$ was higher than 85% for all subjects, as recommended by FDA guidelines [25].

Table 4. Stability of metformin in processed and unprocessed human plasma (n=6)

Storage condition	Concentration added (ng/ml)	Concentration found (ng/ml) (± SD)	Precision (RSD, %)	Accuracy (RE, %)
Processed samples	300	308 (±27.99)	9.1	2.7
24 hr. (room temperature)	800	824.2 (±32.76)	4.0	3.0
48 hr. (-20ºC)	300	299.6 (±10.50)	3.5	0.1
	800	761.7 (±0.00)	0.0	4.8
Unprocessed samples				
24 hr. RT	300	292.7 (±3.1)	1.1	2.4
	800	769.3 (±0.00)	0	3.8
30 days (-20ºC)	300	303.1 (±8.16)	2.7	1.0
	800	739.1 (±30.82)	4.2	7.6
Freeze-thaw at -20ºC (3 cycles)	300	312.2 (±13.09)	4.2	4.1
	800	819.7 (±23.06)	2.8	2.5

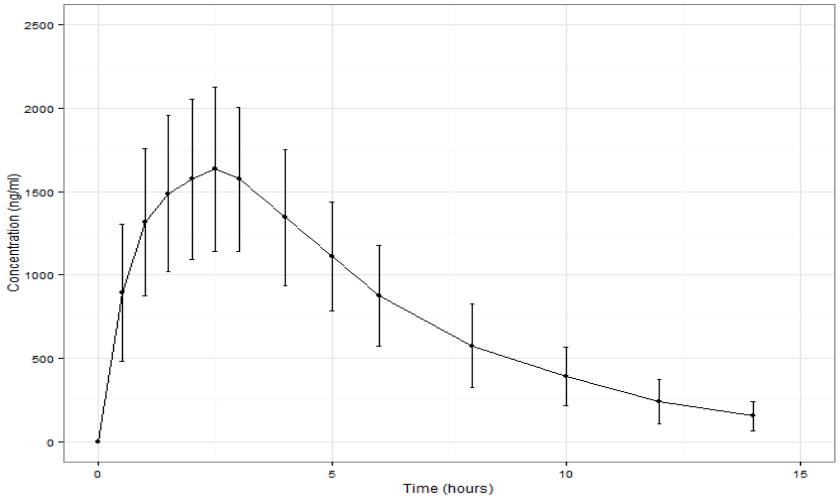

Fig. 3. Mean plasma concentration–time curve of metformin, after oral administration of 850 mg to 20 healthy volunteers. Vertical bars indicate mean standard deviation

Table 5. Mean pharmacokinetic parameters of metformin in human plasma after oral administration of 850 mg tablet

Pharmacokinetic Parameters	Mean Values ± SD (CV%)
Cmax (ng/mL)	1771.750±503.526 (28.420)
AUC_{0-t} (ng h/mL)	10890.663±3220.051 (29.567)
$AUC_{0-\infty}$ (ng h/mL)	11628.992±3586.630 (22.243)
Tmax (h)	2.325±0.654 (28.148)
$T_{1/2}$ (h)	3.11±0.668 (21.481)
Kel (h^{-1})	0.234±0.058 (24.644)

4. CONCLUSION

The optimized HPLC-UV method is selective, accurate, precise and repeatable for the quantification of metformin in plasma samples among healthy volunteers. The run time is short and protein precipitation technique is easy and cost-efficient. The proposed RP-HPLC method, which used a conventional column C-18, is cost effective and less time consuming. This method was also shown to be applicable for research as part of a pharmacokinetic experiment. Even for an injection volume of 20 µl the method is quite sensitive. It can be concluded that the method is suitable for the routine quantification of metformin in human plasma.

CONSENT

Authors declare that 'written informed consent was obtained from the patient for publication of this research paper.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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