Liquid chromatography–tandem mass spectrometry validated method for the estimation of indapamide in human whole blood

Deepak S. Jain [a,b], Gunta Subbaiah [b], Mallika Sanyal [c],
U.C. Pande [a], Pranav Shrivastav [a,*]

[a] *Department of Chemistry, School of Sciences, Gujarat University, Navrangpura, Ahmedabad 380009, India*
[b] *Analytical Development Laboratory, Research Center, Torrent Pharmaceutical Limited, At Village Bhat, Gandhinagar 382428, India*
[c] *Chemistry Department, St. Xaviers' College, Navrangpura, Ahmedabad 380009, India*

Abstract

A highly precise and sensitive method for the estimation of indapamide in human whole blood using high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) is described. The method developed is validated in human whole-blood matrix, with a sensitivity of 0.5 ng/ml as lower limit of quantification. The procedure for the extraction of indapamide and glimepiride as internal standard (IS) involves haemolysis and deprotienation of whole blood using $ZnSO_4$ followed by liquid–liquid extraction using ethyl acetate. The sample extracts after drying were reconstituted and analysed by LC–MS/MS, equipped with turbo ion spray (TIS) source, operating in the positive ion and selective reaction monitoring (SRM) acquisition mode to quantify indapamide in human whole blood. The mean recovery for indapamide was 82.40 and 93.23% for IS. The total run time was 2.5 min to monitor both indapamide and the IS. The response of the LC–MS/MS method for indapamide was linear over the range of 0.5–80.0 ng/ml with correlation coefficient, $r \geq 0.9991$. The coefficient of variance (% CV) at 0.5 ng/ml was 4.02% and the accuracy was well within the accepted limit of $\pm 20\%$ at 0.5 ng/ml and $\pm 15\%$ at all other concentrations in the linear range. This method is fully validated for the accuracy, precision and stability studies and also applied to subject-sample analysis of bioequivalence study for 1.5 mg sustained-release (SR) formulations.
© 2006 Elsevier B.V. All rights reserved.

Keywords: LC–MS/MS; SRM; Indapamide; Human whole blood

1. Introduction

Indapamide [4-chloro-*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide] is an oral, effective and a safe sulfonamide antihypertensive/diuretic drug developed in the early 1970s for the treatment of mild-to-moderate hypertension. Indapamide contains both polar sulfamoyl chlorobenzamide moiety and a lipid-soluble methylindoline moiety. It differs chemically from the thiazides in that it does not possess the thiazide ring system and contains only one sulfonamide group. Unlike other sulfonamides, indapamide has a limited diuretic effect which suggests that its antihypertensive activity might be related more to its vascular properties rather than to its renal effects [1]. During the last 30 years, several classes of antihypertensive drugs have become available, allowing the first-line use of diuretics, β-blockers, ACE inhibitors and calcium antagonists or α-blockers. The recommendations made by all scientific and regulatory authorities have stressed the need for the best efficacy:safety ratio at low dosages of these drugs [2,3]. Diuretics still remain as the first line treatment for essential hypertension because of their long-term beneficial effects on morbidity and mortality and also due to improvements in their use, particularly with the reduction in dosage by using 1.5 mg sustained-release formulations [4,5].

Indapamide is preferentially and reversibly taken up by the erythrocytes in the peripheral blood. The whole-blood to plasma ratio is approximately 6:1 at the time of peak concentration, which decreases to 3.5:1 after 8 h. The C_{max} observed in whole-blood analysis after a single oral dose of 1.5 mg sustained-release formulation is around 17.6 ± 6.3 ng/ml [6]. It is an extensively metabolized drug, with only about 7% of the total dose administered, can be recovered unchanged in the urine during the first 48 h after administration [7]. Also, during actual study sample

* Corresponding author. Tel.: +91 79 26300969.
E-mail address: pranav_shrivastav@yahoo.com (P. Shrivastav).

collection, lysis of red blood cells may lead to haemolysed plasma and hence the actual plasma concentration may not be estimated. To avoid such critical conditions for actual study samples, it is more appropriate to develop and validate the estimation of indapamide in whole blood.

The methods reported in literature for estimation of indapamide in whole blood [6,8–12], serum [8,13,14], plasma [11,12], and urine [11,15–19] involves use of HPLC, or in combination with MS, i.e. LC–MS/MS analytical techniques. These methods had LLOQ between 1.0 and 50.0 ng/ml, applicable for 2.5 mg, 5.0 mg of immediate release and 1.5 mg of sustained-release formulations. Many of these methods [8–11,14], however, do not meet some of the modern drug-development needs with respect to efficient extraction procedure, throughput or sufficient sensitivity. The capability of a single analytical method that encompasses all of the above merits to monitor the total absorption, distribution and elimination pharmacokinetic profile of the pre-clinical and clinical pharmacokinetic studies especially with low-dose sustained-release formulations is of prime concern. The present work aims at achieving all of the above merits.

On account of high blood-to-plasma ratio (6:1), it becomes advantageous to estimate indapamide in whole blood with a very sensitive method for a low dose of 1.5 mg sustained-release formulation. Keeping sensitivity of the method as paramount, a combined reverse phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed to analyse indapamide in whole blood. To the best of our knowledge, this is the first method by LC–MS/MS in whole blood with an LLOQ of 0.5 ng/ml. The MS method is equipped with turbo ion spray source, operating in the positive ion and selective reaction monitoring (SRM) acquisition mode. The whole blood is processed by haemolysis and deprotienation with $ZnSO_4$, followed by liquid–liquid extraction with ethyl acetate. By this method very consistent and reliable low limits of quantification are achieved with high reproducibility and quantitative recoveries. This sensitive method ensures the estimation of indapamide with desired accuracy and precision for elimination-phase concentration in actual subject samples of bioequivalence or bioavailability studies.

2. Experimental

2.1. Materials and instrumental conditions

2.1.1. Reagents

Indapamide as well as glimepiride (IS) was procured from Torrent Research Centre (Ahmedabad, India). Water used for the LC–MS/MS was prepared from Milli Q water purification system (Bangalore, India). Acetonitrile of HPLC grade was purchased from JT Baker (Phillipsburg, USA). Ammonium acetate used for mobile-phase buffer preparation was of molecular-biology grade obtained from Sigma (Steinheim, Germany), while suprapure acetic acid used was procured from Merck (Darmstadt, Germany). Ethyl acetate used for extraction was obtained from Merck (Mumbai, India).

2.1.1.1. Solutions. Ammonium acetate (10 mM) buffer solution was prepared and pH 3.5 was set with acetic acid. Mobile-phase ratio of buffer to acetonitrile was 10:90 (v/v). Control human whole blood was procured from pharmacokinetic unit of Torrent Research Centre (Ahmedabad, India) and was stored at $-70\,^\circ\text{C}$.

2.1.2. Instrumentation

Chromatographic instrument used was Perkin-Elmer PE 200 series pump, PE 200 series auto sampler and PE 200 series column oven, while triple quadrupole mass spectrometer used was API-4000, manufactured by MDS SCIEX, Canada. All of the parameters of LC and MS were controlled by Analyst software version 1.4.

2.1.3. Liquid chromatographic conditions

Perkin-Elmer PE 200 series modules of pump, column oven and autosampler were used for the reverse-phase liquid chromatographic condition settings. Pump was operated at 300 μl/min flow rate; auto sampler temperature was set at $10\,^\circ\text{C}$. Chromatographic column used was from Thermo Electron Corporation, type HyPURITY C18, 100 mm × 2.1 mm (length × inner diameter), with 5 μm particle size and was maintained at $35\,^\circ\text{C}$ in column oven. The mobile phase consisted of buffer (10 mM Ammonium acetate pH 3.5): acetonitrile in ratio of 10:90 (v/v). The LC run time was 2.5 min.

2.1.4. Mass spectrometric conditions

Ionization of indapamide and glimepiride (IS) was carried out using electro spray ionization technique (TIS interface of the API 4000) with positive polarity and multiple reaction monitoring (MRM) mode to monitor the ions with m/z of 366.10 (parent ion) and 132.20 (product ion) (Fig. 1) for indapamide and 491.23 (parent ion) and 352.3 (product ion) for IS. For both indapamide and IS, the source parameters maintained were Gas 1 (GS1): 30.0 psig, Gas 2 (GS2): 40.0 psig, ion spray voltage (IS): 5500.0 V, turbo heater temperature (TEM): $400.0\,^\circ\text{C}$, interface heater (Ihe): ON, entrance potential (EP): 10.0 V, collision activation dissociation (CAD): 4 psig, curtain gas (CUR): 20 psig, while the declustering potential (DP), collision energy (CE) and cell exit potential (CXP) applied was 64, 19.5 and 7 V, respectively for indapamide and 65, 18 and 8.5 V, respectively for IS. The indapamide analysis data were acquired and quantified using analyst classic of Analyst software version 1.4.
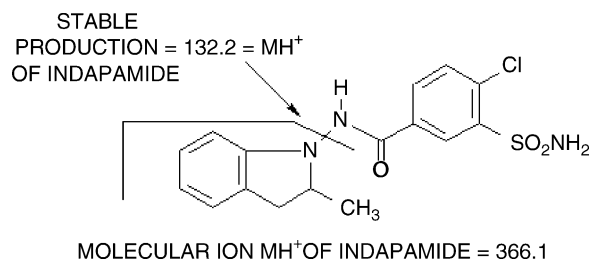

Fig. 1. Structure of indaparnide with probable product ion.

2.1.5. Preparation of standard stocks and blood samples (control and standards)

The standard stock solutions of 100 µg/ml for indapamide and glimepiride (IS) were prepared by dissolving their requisite amount in methanol. These stock solutions were further diluted appropriately to get an intermediate concentration of 10 µg/ml for indapamide and IS, respectively. Working solution of indapamide of different concentrations required for spiking blood calibration, and quality control samples were subsequently prepared using the standard and intermediate stock solutions. IS working solution of 250 ng/ml was prepared using the intermediate stock of 10 µg/ml and was used as internal standard in blood samples preparation. All of the standard stock, intermediate stock and working stock solutions were prepared and stored at 2–8 °C until use. Drug free blood, i.e. control (blank) blood was withdrawn from the deep freezer and allowed to get completely thawed before use. Five percent spiking with working stock solution of indapamide was done in blank blood to achieve the desired concentration of indapamide for calibration and quality control (QC) samples. The spiked QC samples were stored at −70 °C for stability studies.

2.1.6. Sample extraction

The control samples of human whole blood were taken out from −70 °C freezer and kept at room temperature for 30–45 min for thawing. The samples were vortexed adequately using a vortex mixer before pipetting. Using a micropipette, 0.5 ml of blood was transferred into 15 ml capacity glass centrifuge tube, to which 50 µl of working solution of IS was added. To ensure the complete release of indapamide bound to red blood cells, to the same tube, 500 µl of 5.0% ZnSO₄ solution was added and vortexed (using Multi-Pulse Vortexer) thereafter for 1 min. Further, 50 µl of 0.1 M NaOH and 5 ml of ethyl acetate was added and vortexed for another 5 min. The tube was then centrifuged at $2602 \times g$ for 5 min. Out of 5 ml of ethyl acetate, 4 ml was transferred to another tube for drying in Zymark LV evaporator maintained at 40 °C under a stream of nitrogen. The residue obtained after drying was reconstituted with 250 µl of water: acetonitrile (10:90) mixture and loaded in the autosampler vial. Only 5 µl of the reconstituted volume was then injected into the column in partial loop mode.

3. Results

3.1. Selectivity and sensitivity

The above rapid method of extraction gave very good selectivity and sensitivity for the analysis of indapamide and IS in the blank blood. The retention time (RT) was short for both which makes it suitable for routine analysis. This method gave clean chromatograms free of background interference. Test for selectivity (specificity) was carried out in different lots of blank blood. Four different lots of heparinised blood, one lot of lipemic blood and one lot of haemolysed blood were chromatographed. The area observed at the elution time of indapamide was 7% or less

relative to the LLOQ in six blank blood samples tested, whereas no area was observed at the elution time of IS. The % CV calculated for the indapamide area of LLOQ and area of IS are found to be 6.11 and 2.37, respectively. The sensitivity results of intra-assay and inter-assay accuracy and precision at LLOQ level are shown in Table 2. The aim of performing selectivity (specificity) check with these different types of blood samples is to ensure the quality of the results of study sample analysis. Fig. 2 represents the blank blood specificity, zero standard (blood sample processed with internal standard only) and the peak response of indapamide at LLOQ (0.5 ng/ml).

3.2. Linearity

The linearity of the method was determined by the analysis of standard plot associated with a nine-point standard calibration curve. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of indapamide was calculated from the simple linear equation using regression analysis of spiked blood calibration standard with the reciprocity of the drug concentration as a weighing factor (1/concentration, i.e. $1/x$); $y = mx + c$.

The calibration curves were linear from 0.5 to 80.0 ng/ml with correlation coefficient of $r \geq 0.9991$. The R.S.D. of 'slope' of five linearities was only 3.92% while that for 'r' was only 0.03%. The observed mean back calculated concentrations with accuracy (%) and precision (% CV) of six linearities are given in Table 1.

3.3. Recovery

The percentage recovery of indapamide was determined by comparing the mean area of five replicates each of extracted quality control samples; low quality control (LQC = 1.5 ng/ml), middle quality control (MQC = 24.0 ng/ml) and high quality control (HQC = 56.0 ng/ml) samples with mean area of freshly prepared un-extracted LQC, MQC and HQC samples (i.e. proportionate spiking of QC's and IS in extracted blank blood-reconstituted solution to get the concentration equivalent to the extracted QC concentration). Thus, the overall mean recovery for indapamide at LQC, MQC and HQC was 81.71, 83.69 and 81.80%, respectively and variability (% CV) within their replicates was 1.88, 1.60 and 2.92, respectively and between them (i.e. recoveries at these three different level) was only 1.36%. The recovery of IS was found to be 93.23% with CV of 2.98%. Thus, the consistency (precision calculated as % CV) in quantitative recoveries of indapamide and IS authenticates the extraction procedure for its application to routine sample analysis.

3.4. Precision and accuracy

The intra-assay precision and accuracy was calculated for five replicates at each LLOQ, LQC, MQC and HQC levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The results are given in Table 2.
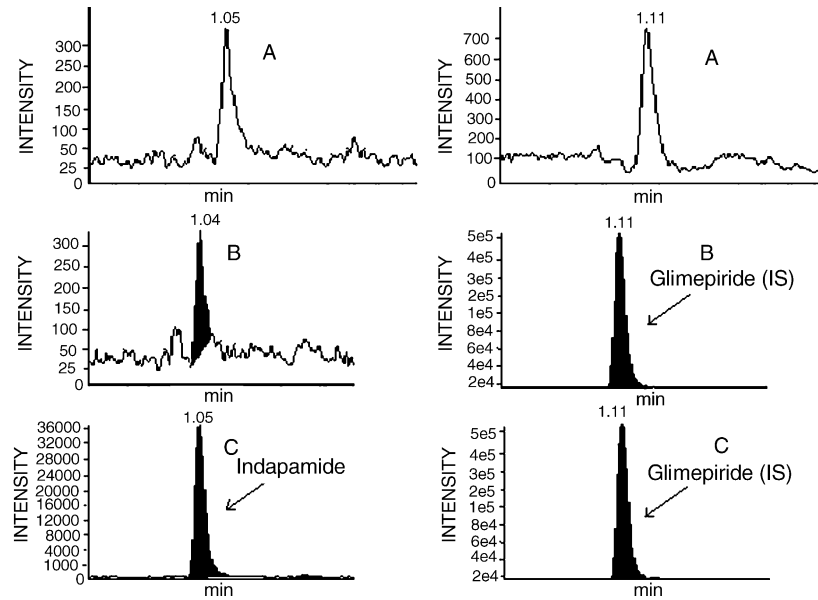
Fig. 2. (A) Representative chrornatooiam of control blood; (B) zero standard; (C) LLOQ (0.5 ng/ml); *Y*-axis represents intensity in CPS; *X*-axis represents time in min.

Table 1
Back calculated concentration of calibration standards (CS) from each calibration curve of indapamide

Linearity	Concentration found (ng/ml)								
	CS-1 0.5[a]	CS-2 1[a]	CS-3 4[a]	CS-4 8[a]	CS-5 16[a]	CS-6 32[a]	CS-7 48[a]	CS-8 64[a]	CS-9 80[a]
1	0.508	0.997	3.999	8.248	15.424	30.617	49.481	64.263	79.962
2	0.483	0.990	4.135	8.450	16.072	29.025	48.914	66.080	79.352
3	0.500	0.967	4.054	8.221	15.939	31.582	49.059	63.209	79.969
4	0.458	0.944	4.047	8.694	17.185	31.838	47.621	63.049	79.664
5	0.518	0.921	4.043	8.226	16.103	32.018	48.883	62.117	80.671
6	0.484	1.002	4.064	8.258	15.823	32.023	47.115	64.414	80.317
n	6	6	6	6	6	6	6	6	6
Mean	0.492	0.97	4.057	8.35	16.091	31.184	48.512	63.855	79.989
Accuracy (%)	98.367	97.017	101.43	104.37	100.57	97.449	101.07	99.774	99.986
S.D.	0.021	0.032	0.044	0.189	0.589	1.181	0.926	1.38	0.466
CV (%)	4.36	3.34	1.09	2.27	3.66	3.79	1.91	2.16	0.58

[a] Added concentration (ng/ml).

3.5. Matrix effect

Matrix effect was checked with total six different lots of blood which included five lots of normal control heparinised blood and one lot of lipemic control heparinised blood. Three samples each of LQC and HQC were prepared from different lots of blood (in total 36 QC samples) and checked for the inaccuracy in all of the QC samples. This was performed with the aim to see the matrix

Table 2
Indapamide intra-assay and inter-assay accuracy and precision

Quality control samples	Concentration added (ng/ml)	*n*	Intra-assay				*n*	Inter-assay			
			Mean concentration found (ng/ml)[a]	RE (%)	S.D.	CV (%)		Mean concentration found (ng/ml)[b]	RE (%)	S.D.	CV (%)
LLOQ	0.5	5	0.514	2.8	0.021	4.02	15	0.492	1.55	0.031	6.26
LCQ	1.5	5	1.523	1.5	0.059	3.90	15	1.534	2.28	0.055	3.90
MQC	24	5	23.199	−3.3	0.765	3.30	15	24.027	0.11	0.882	3.67
HQC	56	5	53.452	−4.6	1.267	2.37	15	57.512	2.7	3.712	6.45

S.D., standard deviation, RE, relative error, CV, coefficient of variance, *n*, total number of observations for each concentration.
[a] Mean of five replicate observations at each concentration.
[b] Mean of 15 observations recorded over three different analytical runs (5 replicates/run).

Table 3
Matrix effect in six different lots of heparinised whole blood

	LQC (1.5 ng/ml)[a]						HQC (56 ng/ml)[a]					
	lot-1	lot-2	lot-3	lot-4	lot-5	lot-6	lot-1	lot-2	lot-3	lot-4	lot-5	lot-6
Mean calculated concentration (ng/ml)	1.47	1.44	1.50	1.44	1.45	1.42	52.88	50.34	51.91	51.85	51.75	51.18
% Inaccuracy	−1.93	−3.84	0.13	−4.07	−3.20	−5.20	−5.57	−10.11	−7.30	−7.42	−7.59	−8.61

[a] Three samples for each lot at each concentration were prepared.

effect of these different lots of blood on the back calculated value of QC's nominal concentration and all the results found were well within the acceptable limit of ±15%. Hence, this clearly proved that the elution of endogenous matrix peaks in the dead volume time does not affect the quantitation of indapamide and IS peak. Therefore, the method of extraction of indapamide from whole blood was rugged enough and gave accurate and consistent results when applied to real patient samples. The details of the performed matrix effect experiment are summarized in Table 3.

3.6. Stability study

Stability experiments were performed very exhaustively with this method to evaluate the indapamide stability in stocks solutions and in blood samples under different conditions. The conditions which occurred during actual study sample analysis were simulated in method validation stability studies, such as stock solution stability of indapamide and IS, stability in blood at room temperature, extracted sample stability (dry-state stability at 2–8 °C and process stability at 10 °C), freeze thaw stability and long-term stability at −70 °C. The results obtained were well within the acceptable limit. IS stock solution was also found to be stable.

Stock solutions of indapamide and IS were stable at room temperature for 24 h and at 2–8 °C for 48 h and their mean percent change was within ±5%. Indapamide in control human blood at room temperature was stable for 6 h at least with mean percent change of −1.57%. Indapamide in the dried residue was stable for 20 h at 2–8 °C while the reconstituted extract in autosampler at 10 °C was found to be stable for up to 43 h (process stability) with mean percent change of 0.62 and −1.76%. Indapamide was found to be stable for at least three freeze and thaw cycles with mean percent change of 4.10%. The indapamide spiked blood samples stored at −70 °C for long-term stability experiment were found stable for at least 40 days with mean percent change of 0.77%.
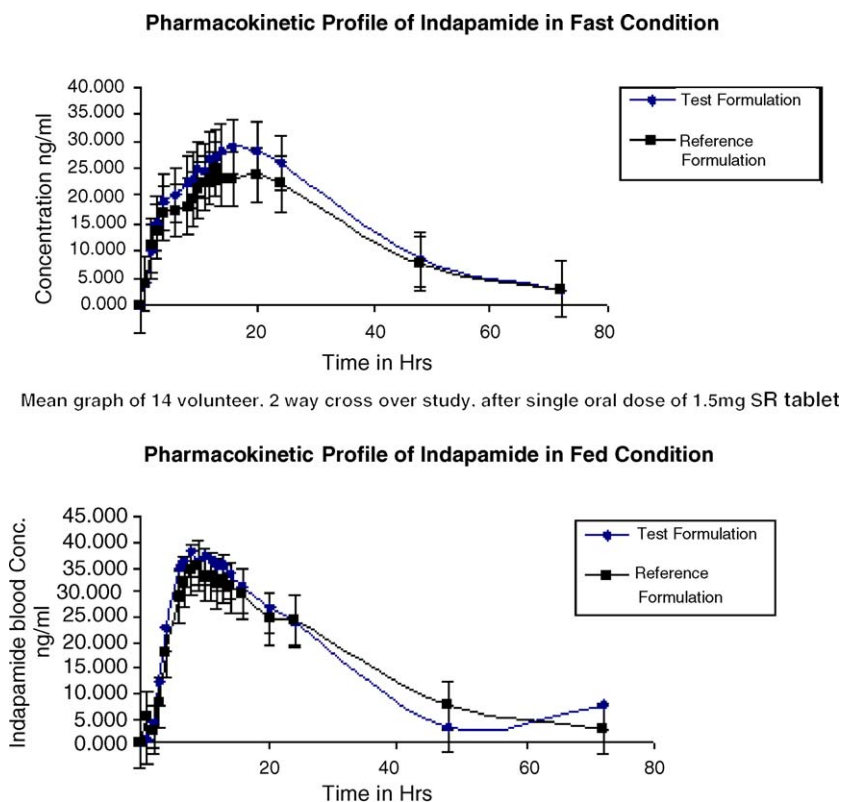

Fig. 3. Mean graph of 14 volunteer, two way cross over study, after single oral dose of 1.5 mg SR tablet.

3.7. Application of method

The above validated method was successfully used for the assay of indapamide in human subject samples who received 1.5 mg sustained-release test and reference formulations under fasting and fed conditions. The design of the study comprised "a randomized, open label, two period, two treatment, two sequence crossover bioequivalence study of indapamide 1.5 mg sustained-release formulation in 14 healthy human subjects under fasting and fed condition". The method was sensitive enough to monitor the indapamide blood concentration up to 72 h. In totality approximately 1500 samples including the calibration and QC samples with volunteer samples were run in only 8 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The mean pharmacokinetic profile for both the treatment, under fasting and fed condition is presented in Fig. 3.

4. Results and discussion

The objective of this work was to develop a highly sensitive method to estimate indapamide in human whole blood, especially in the absorption and elimination phase after oral administration of 1.5 mg sustained-release formulation. It was a challenge for us to develop a method for the quantitative extraction of indapamide, bound to the red blood cells. Many trials of liquid–liquid extraction of indapamide from whole blood were carried out using different solvents, viz. diethyl ether, *tert*-butyl methyl ether (TBME), TBME and hexane, etc., but all resulted in inconsistent recoveries and poor sensitivity. In an attempt to get cleaner extracts, the RBCs were first ruptured by water and then loaded on SPE cartridge, but it was found that the cartridge got blocked. Alternately, the precipitation of blood proteins and RBCs was tried with acetonitrile; however, the desired and consistent sensitivity and recoveries could not be achieved at low levels. Finally, quantitative and consistent recovery was obtained by rupturing the RBCs at $-70\,^\circ\mathrm{C}$, and further haemolysis and precipitation of RBC was done using a hypertonic solution of $ZnSO_4$, followed by liquid–liquid extraction with ethyl acetate. The basic underlying advantage of this optimized method is that it utilizes only 0.5 ml of blood whereby avoiding the bleeding out of 5–6 ml of blood to obtain 3 ml of plasma. Also, the method involves minimum usage of organic solvent, drying and reconstitution in 250 μl, and only 5 μl of the reconstituted volume can be directly injected into LC–MS. This method gives very good intra-assay and inter-assay precision and accuracy with quantitative recovery. The short retention time of 1.05 and 1.12 min for indapamide and glimepiride (IS), respectively, gives the total run time of 2.5 min only. The method was more selective because the data acquisition was performed with MRM mode, and thus the need to separate indapamide and internal standard chromatographically is not mandatory. From the results of all of the validation parameters and the application of the method to subject samples analysis, we conclude that the above validated method can be highly useful for the therapeutic drug monitoring both for analysis of routine samples of single dose or multiple dose pharmacokinetics and for the clinical trial samples with precision, accuracy and high throughput. A further advantage of this method observed and proved is that the limit of quantification is low enough to monitor at least five half-lives of indapamide concentration with good inter-assay reproducibility (CV) for the quality controls run with patient samples

References

[1] D.B. Campbell, A.R. Taylor, R.A. Moore, Brit. J. Clin. Pharmacol. 3 (1976) 971.
[2] The sixth report of the Joint National Committee on prevention, detection, evaluation and treatment of high blood pressure (JNC VI). Arch. Intern. Med. 157 (1997) 2413.
[3] 1993 guidelines for the management of mild hypertension. Memorandum from World Health Organization/International Society of Hypertension Meeting, Guidelines Subcommittee of the WHO/ISH Mild Hypertension Liaison Committee, Hypertension 22 (1993) 392.
[4] R. Collins, R. Peto, S. MacMahon, Lancet 335 (1990) 827.
[5] Medical Research Council trial of treatment of hypertension in older adults: principal results. BMJ 302 (1992) 405.
[6] P. Schiavi, R. Jochemsen, D. Guez, Fundam. Clin. Pharmacol. 14 (2000) 139.
[7] M.J. Burke, S.H. Preskorn, in: F.E., Bloom, D.J., Kupfer (Eds.), Psychopharmacology: Physicians' Drug References. Medical Economic Company, Oradell, NJ, 44th ed., 2004, p. 2202.
[8] D. Zendelovska, T. Stafilov, M. Stefova, J. Chromatogr. B 788 (2003) 199.
[9] V. Hutt, G. Pabst, C. Dilger, G. Poli, D. Acerbi, Eur. J. Drug Metab. Pharmakokinet. 19 (1994) 59.
[10] R.B. Miller, D. Dadgar, M. Lalande, J. Chromatogr. 614 (1993) 293.
[11] R.L. Choi, M. Rosenberg, P.E. Grebow, J. Chromatogr. 230 (1982) 181.
[12] P.E. Grebow, J.A. Treitman, A.K. Yeung, M.M. Johnston, J. Pharm. Sci. 70 (1981) 306.
[13] F. Albu, C. Georgita, V. David, A. Medvedovici, J. Chromatogr. B 816 (2005) 35.
[14] D. Chen, Zhongguo Yi Xue Ke Xue Yuan Xue Bao. 12 (1990) 286.
[15] K. Deventer, F.T. Delbeke, K. Roels, P.V. Eenoo, Biomed. Chromatogr. 16 (2002) 529.
[16] D. Thieme, J. Grosse, R. Lang, R.K. Mueller, A. Wahl, J. Chromatogr. B 757 (2001) 49.
[17] M.J. Legorburu, R.M. Alonso, R.M. Jimenez, E. Ortiz, J. Chromatogr. Sci. 37 (1999) 283.
[18] P. Pietta, A. Calatroni, A. Rava, J. Chromatogr. 228 (1982) 377.
[19] P.E. Grebow, J.A. Treitman, A.K. Yeung, J. Pharm. Sci. 67 (1978) 1117.