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Development and validation of bioanalytical method for simultaneous estimation of ramipril and hydrochlorothiazide in human plasma using liquid chromatography-tandem mass spectrometry

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ABSTRACT

The present study describes a novel liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for the simultaneous estimation of ramipril (RAM) and hydrochlorothiazide (HCTZ) in human plasma using liquid–liquid extraction technique. This method made use of electrospray ionization in positive mode for RAM and in negative mode for HCTZ using triple quadrupole mass spectrometry where carbamazepine was used as an internal standard (IS). Analytes were recovered by methyl tertiary butyl ether:dichloromethane (85:15) subsequently separated on an Enable C_{18} G column (150 mm × 4.6 mm, 5 μm) using methanol:0.1% formic acid in water (85:15) as a mobile phase, at a flow rate of 0.5 mL/min. Quantification of RAM, HCTZ and IS was performed using multi-reaction monitoring mode (MRM) where transition of m/z 417.2 → 234.1 (RAM) and 237.0 → 194.0 (IS) in positive mode and 296.1 → 205.0 for HCTZ in negative mode. The calibration curve was linear ($r^2 > 0.99$) over the concentration range of 2–170 ng/mL for RAM and 8–680 ng/mL for HCTZ. The intra-day and inter-day precisions were <15% and the accuracy was all within ±15% (at LLOQ level ±20%). Additionally, the LC-MS/MS method was fully validated for all the other parameters such as selectivity, matrix effect, recovery and stability as well. In conclusion, the findings of the present study revealed the selectivity and sensitivity of this method for the simultaneous estimation of RAM and HCTZ in human plasma.

1. Introduction

Ramipril (RAM), an angiotensin converting enzyme (ACE) inhibitor, is being used in treatment of hypertension and heart failure. Ramipril, (2*S*,3a*S*,6a*S*)-1-{*N*-[(*S*)-1-ethoxycarbonyl-3-phenylpropyl]L-alanyl}perhydrocyclopenta[*b*]pyrrole-2-carboxylic acid, converted into an active moiety, i.e. di-acid ramiprilate by hepatic esterase which cleaves ester moiety to set free di-acid ramiprilate [1,2]. Many researchers demonstrated several techniques for qualitative and quantitative determination of RAM in the biological fluids using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [3–5], high performance liquid chromatography (HPLC) [6] and spectrophotometry methods [7]. Further, stability testing of RAM in pharmaceutical preparations has been studied using high performance thin layer chromatography (HPTLC) [8].

Hydrochlorothiazide (HCTZ), 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide, is an anti-diuretic and anti-hypertensive drug. It acts via inhibiting Na^+–Cl^- symporter in the distal convoluted tubule and thereby reducing extracellular fluid volume, leading to fall in cardiac output which produces vasodilatation [9,10]. HCTZ has been evaluated in biological fluids and pharmaceutical preparations using LC-MS/MS [11–14] and HPLC [15,16], HPTLC, respectively [17].

Although, RAM and HCTZ have been studied in pharmaceutical preparations as well as in biological fluids using diverse analytical techniques, simultaneous estimation of these two drugs has got little attention. Moreover, RAM and HCTZ produce different ionization pattern therefore, it is required to use ionization source which can produce both the ionization with very less switching time in LC-MS/MS. Therefore, the present study was designed to develop and validate a method for simultaneous determination of RAM and HCTZ in human plasma using LC-MS/MS.

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2. Materials and methods

2.1. Chemicals and reagents

RAM, HCTZ and IS were kindly provided by Cadila Pharmaceuticals Ltd., Ahmedabad, India. Human Plasma was generously donated by Rajkot Voluntary Blood Bank and Research Centre, Rajkot, Gujarat, India. Methanol and acetonitrile of HPLC grade were purchased from Spectrochem Pvt. Ltd., Mumbai, India. Formic acid was purchased from Merck Millipore, Mumbai, India. Dichloromethane and methyl tertiary butyl ether were of HPLC grade and purchased from Spectrochem Pvt. Ltd., Mumbai, India. Water was purified using a Milli-Q water purification system, Millipore Pvt. Ltd., Ahmedabad, Gujarat, India.

2.2. Instrumentation

The LC-MS/MS system consisted of a Shimadzu liquid chromatography system (Shimadzu Corporation, Japan) which consisted of a LC-20AD solvent delivery system, a DGU-20A5R vacuum degasser, a CTO-20AC thermostated column oven and SIL-20AC autosampler, and coupled with a triple quadrupole mass spectrometer LCMS-8030 (Shimadzu Corporation, Japan). Data acquisition and processing were performed using Lab solution software (version 5.53 SP3C) from Shimadzu Corporation, Japan.

2.3. Chromatographic conditions

Chromatographic separations were achieved on an Enable C_{18} G column (150 mm × 4.6 mm, 5 μm pore size) placed in thermostated column oven at 40 °C using mobile phase consisting of 85% of methanol and 15% formic acid in water (0.1%, v/v), at a flow rate of 0.5 mL/min. Sample injection volume was 10 μL. Analytical run time was 6 min.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in positive and negative ion mode using electrospray ionization (ESI) source. Tuning parameters were optimized for RAM, HCTZ and IS by infusing a solution containing 250 ng/mL of each analytes. Nitrogen was used as nebulizing (3 L/min) and drying gas (15 L/min). Spray voltage and interface temperature were regulated at 4500 V and 350 °C, respectively throughout the study. For collision induced dissociation (CID), argon was used as collision gas at a pressure of 2.5 kPa. Quantification of analytes performed using multiple reaction monitoring of the transitions m/z 417.2 → m/z 234.1 for RAM, m/z 237.0 → m/z 194.0 for IS in positive mode and m/z 296.1 → 205.0 for HCTZ in negative mode, with the dwell time of 200 ms per transition. Optimized collision energies of −25, 25 and 25 eV were used for RAM, HCTZ and IS, respectively.

2.5. Preparation of standard and quality control (QC) samples

The standard stock solutions of RAM, IS and HCTZ were prepared by dissolving 10 mg of analytes in methanol to give final concentration of 1000 μg/mL for each analyte. These solutions were further diluted with methanol to give final concentration of 10 and 40 μg/mL of RAM and HCTZ, respectively. Further, solutions were diluted with methanol to achieve working standard solutions at the concentrations of 0.040, 0.080, 0.170, 0.340, 0.680, 1.360, 2.040, 2.720, 3.400 μg/mL for RAM and 0.160, 0.320, 0.680, 1.360, 2.720, 5.440, 8.160, 10.880, 13.600 μg/mL for HCTZ. A working solution of IS was prepared by diluting the standard stock solution of carbamazepine in methanol to achieve a final concentration of 50 μg/mL. The working standard solutions (10 μL) were used to spike blank human plasma sample (200 μL) to build up the calibration curve of both the analytes and for quality control in validation studies [18]. The final concentrations in standard plasma samples were 2, 4, 8.5, 17, 34, 68, 102, 136, 170 ng/mL for RAM and 8, 16, 34, 68, 136, 272, 408, 544, and 680 ng/mL for HCTZ. The QC samples were prepared in the same way as the calibration samples. The plasma concentrations of QC samples were 2, 6, 70, 140 ng/mL for RAM and 8, 24, 280, 560 ng/mL for HCTZ. All samples were stored at 4 °C until analysis.

2.6. Plasma sample preparation

Aliquots of 200 μL of unknown plasma, blank, calibration curve standard (CCs) and quality control standard (QCs) samples were prepared in 2 mL eppendorf centrifuge tubes and mixed with 100 μL of buffer solution (0.5 M HCl) to which 50 μL of IS solution was added. Subsequently, 1 mL of liquid extraction mixture (methyl tertiary butyl ether:dichloromethane; 85:15) was added to the above solutions and vortexed for 10 min. The resultant mixture was then centrifuged at 6000 rpm for 10 min and 0.8 mL of supernatant thus obtained was transferred to glass vials, evaporated to dryness in a vacuum oven at 40 °C under the gentle stream of nitrogen. The dried samples were reconstituted by addition of 100 μL of mobile phase, loaded into autosampler and then 10 μL of reconstituted samples were injected into LC-MS/MS system.

2.7. Quantification

Quantitative analysis of RAM and HCTZ was performed using carbamazepine as an IS. Calibration curves were established with CCs prepared in plasma. Nine-point CCs constructed using peak area ratio of analytes to IS. Concentration of analytes in QCs and unknown samples were calculated by interpolation from the calibration curves.

2.8. Method validation

Method validation protocol was based on the recommendations of the United States Food and Drugs Administration (USFDA) guidelines [18].

2.8.1. Selectivity

The selectivity of method to ward endogenous plasma matrix components was assessed by comparing the interfering signals in 10 different batches of plasma (seven were of K_3EDTA, and one each of lipidemic, haemolyzed and heparinized plasma) with the signals of analytes and IS. Aliquots of plasma samples were used to prepare lower limit of quantification (LLOQ) and blank samples. Baseline noise should be <20% of analyte response at this concentration level.

2.8.2. Linearity, accuracy and precision

The linearity of the method was assessed by processing a nine-point calibration curve over the concentration range of 2–170 ng/mL for RAM and 8–680 ng/mL for HCTZ in three consecutive runs. Calibration curves were constructed by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte to IS. Each calibration curve was analyzed individually by least square weighted ($1/x^2$) liner regression. The inter- or intra-batch accuracy and precision were evaluated using six replicates of QC samples at LLOQ, lower (LQC), middle (MQC) and higher (HQC) concentration levels for three consecutive analytical days. The concentration of QC samples was selected from the calibration curve range. The criteria for acceptability of the data included precision within 15% coefficient of variance (% CV) and an accuracy within ±15% relative error (% RE) of the nominal values. Lower limit of quantification was determined with acceptable precision