Development and validation of bioanalytical method for the determination of valsartan in human plasma

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Abstract:

A bioanalytical method which utilizes high performance liquid chromatography with mass spectrometry method has been developed and validated for the quantification of valsartan in human plasma. The samples were processed by precipitation with formic acid then extracted with diethyl ether. Benazepril was used as an internal standard. The chromatographic separation is performed through C18 column with a mobile phase consisting of deionized water, acetonitrile and formic acid, followed by mass spectrometric detection in the positive ionization mode. The proposed method was specific and had been validated in the linear range of 50.0 – 5000.0 ng/ml for valsartan. The validation results were as follows: the intra-day and inter-day precision were 3.46 to 8.33% and 5.85 to 7.05% respectively, the intra-day and inter-day accuracy were 93.53 to 107.13% and 95.26 to 104.0% respectively. The recovery for valsartan and benazepril was 81.4% and 113.7% respectively. Also, stability was studied and the results obtained for short-term stability 99.24 to 102.32%, for freeze / thaw stability 99.75 to 99.95% and for long-term stability 98.24 to 103.03%. It can be concluded that the method can be applied in pharmacokinetic bioequivalence studies.

Key words: Liquid chromatography, mass spectrometry, validation, quantification, valsartan.

تطوير و تقييم صلاحية طريقة تحليل لقياس تركيز فالسارتان في بلازما دم الانسان

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الخلاصة:

تم تطوير طريقة تقييم كمي لفارسارتان في بلازما الإنسان بإستخدام منظومة الكروماتوغرافيا السائل عالي الأداء مع الكشف بكاشف مطياف الكتلة , و تقييم صلاحيتها ، حيث تم تحضير العينات إعتمادا على الترسيب بحامض الفورميك ثم الإستخلاص بثنائي أثيل إيثر ، كما أستخدم بينازبريل كمعاير داخلي. أجريت عملية الفصل الكروماتوغرافي خلال عمود نوع سي 18 مع طور متحرك يتكون من ماء خالي من الأيونات و أسيتونتريل و حامض الفورميك ، وبعدها الكشف بمطياف الكتلة في الشكل أيون الموجب . تم إثبات صلاحية الطريقة بأنها محددة ضمن المدى الخطي لها من 50 إلى 5000 نانوغرام فالسارتان لكل مليلتر. نتائج التقييم كانت كالأتي: الإنضباط في ذات اليوم و بين الأيام يتراوح من 3,46 إلى 8,33٪ و من 5,85 إلى 7,05٪ على التوالي , الدقة في ذات اليوم و بين الأيام تتراوح من 93,53 إلى 107,13٪ على التوالي , بالإضافة إلى ذلك , كانت النتائج للثباتية في الفترة القصيرة تتراوح من 99,24 إلى 102,32٪ و الثباتية بعد التجميد ثم الإسالة تتراوح من 99,75 إلى 99,95٪ , و أخيرا الثباتية في الفترة الطويلة تتراوح من 98,24 إلى 103,03٪ . تشير هذه النتائج إلى إمكانية تطبيق هذه الطريقة في تحليل العينات المستخدمة في دراسات التكافؤ الحيوي لحركية الدواء.

الكلمات المفتاحية: الكروماتوغرافيا السائل , مطياف الكتلة , تقييم صلاحية , تقييم كمي , فالسارتان

Introduction

Bioanalytical method employed for the quantitative determination of drugs and their metabolites in biological fluids and its validation is essential for the evaluation and the interpretation of bioava-ilability, bioequivalence, pharma-cokinetic and toxico-kinetic study data [1]. When a method is developed, with the desired attributes, it should be validated to meet its objectives and standards [2]. This is because "Incurred" or study samples vary in their composition compared to the standards and quality control samples which are used for the validation of the method [3].

Sample preparation is a critical step in bioanalytical method development and validation. This process aims to remove interferences and to make the sample with the higher concentration of analyte which contributes to the sensitivity of the method [4]. A various method can be utilized, including: Protein Precipitation, Liquid-Liquid Extraction, Solid Phase Extraction, Solid Phase Microextraction, Matrix Solid-Phase Dispersion and Supercritical Fluid Extraction [4].

Coupling of separation technique such as liquid chromatography (LC) with mass spectrometry (MS), permits a fast quantitative determination of molecules in complex biological mixtures such as plasma [5]. The sensitivity, selectivity, and speed of MS turned to be a superb solution for pharmacokinetics applications. Speed of analysis is contributed to the discovery and development of drug candidates, which impacted the overall time required for developing new medicines [6].

Valsartan is an angiotensin-receptor blocking drug, used in the treatment of hypertension. It has the empirical formula $C_{24}H_{29}N_5O_3$ with a molecular weight of 435.52 g/mol [7]. Valsartan is freely soluble in methanol, acetonitrile and sparingly soluble in water. Since valsartan structure contains acid (pKa=4.73) and carboxylic groups (Figure 1), absorption is influenced by pH along the gastrointestinal tract [7].

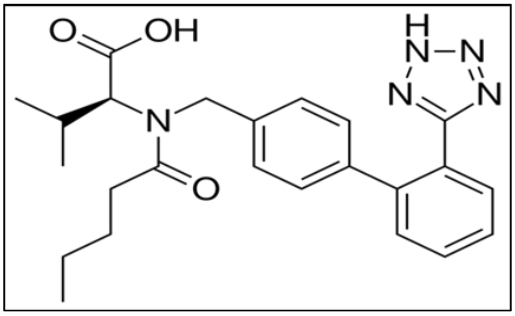

Figure (1):Structure of valsartan

The partition coefficient of valsartan is 0.033 (log P=1.499), so that it is hydrophilic at physiological pH [8]. In the biopharmaceutical classification system, valsartan has been classified as Class III drug with low permeability and high solubility [9]. Valsartan bioavailability is about 25% due to its acidic properties which render the drug poorly soluble at low pH and is absorbed from the upper part of GIT that is acidic in nature and where its solubility is low [10].

Materials

Acetonitrile analytical grade (Scharlua), formic acid analytical grade (Scharlua), diethyl ether analytical grade (Scharlua), human plasma, De-ionized water, valsartan reference standard (USP), Benazepril (Internal Standard), 0.45 µm membrane filters, 10 ml glass tubes. Symmetry C18 (4.6 mm x 50 mm, 3.5 µm) column, 1.50 ml micro-centrifuge tubes (Eppendorf).

biological matrix of plasma. Stability data are given in Table7.

Experiment

Method development

Stock solutions of valsartan reference standard in human plasma was prepared, then diluted to obtain 50, 100, 200, 500, 1000, 2000, 3000 and 5000 ng/mL of valsartan for the construction of calibration curves, with 150, 2500 and 4250 ng/mL as quality control (QC) samples. Benazepril concentration as internal standard was 2500

ng/mL A 500 µl aliquot from each solution was processed by addition of 200 µl of 10% formic acid, vortexed for one minute, then 500 µl of diethyl ether was added, mixed for five minutes, followed by centrifuge at 10000 rpm for five minutes, the upper layer was evaporated at 40°C under nitrogen stream. The residue was reconstituted with 500 µl of the mobile phase, 10 µl injected into LC/MS system. The mobile phase composed of deionized water: acetonitrile (20: 80 v/v) and 0.2% formic acid, in isocratic condition through C18 (4.6 mm x 50 mm, 3.5 µm) column. The mass detector was set in the positive ionization mode m/z = 435.53 for valsartan and m/z = 424.50 for benazepril [11].

Method validation

The method was validated according to the current international approach for bio-analytical method validation, which satisfies the requirements of statutes and regulations [12]; accordingly, the method validation was evaluated in terms of:

Specificity

The specificity of the method was determined through the screening for three batches of controlled human blank plasma. Specificity can be verified if no co-eluted peak from endogenous plasma components is seen the retention times of the drug and the internal standard.

Linearity

For the determination of linearity, standard calibration curves of 8 points were prepared. In each of six days, a calibration curve was prepared and its fit was calculated by weighted least square linear regression equation. In this method, valsartan determination in human plasma was constructed in the concentrations range from 50 to 5000 ng/mL. The least-squares linear regression equation ($y = b\chi + a$), of the best-fit peak area ratios versus concentration was used to back-calculate the concentrations, where b is the slope, a is the intercept, y represents the peak area of valsartan / peak area of benazepril and χ represents the concentration of valsartan.

Accuracy and Precision

Accuracy and precision were held over the first three days of the validation course. Quality control (QC) samples were calculated by employing the regression of the calibration curve that was carried out at the same day. The accuracy and precision deviation values should be within 15% of the nominal values. A statistical summary for mean, standard deviation, coefficient of variation and accuracy was calculated.

Intra-day accuracy and precision

The intra-day accuracy and precision of the assay were measured by analyzing twelve QC samples at each concentration level (150, 2500 and 4250 ng/mL); then their concentrations were back calculated. The deviation of the mean from the nominal value is the measure of accuracy.

Inter-day accuracy and precision

The inter-day accuracy and precision were investigated at each concentration level (150, 2500 and 4250 ng/mL) over three days. Analysis was carried out using twenty-four QC samples at each level (twelve QC at day one and six QC per each day of the next two days).

Recovery

The detector response of QC samples was compared to the detector response of an equivalent pure authentic standard solution reconstituted to contain valsartan and benazepril concentrations assuming 100%. The recoveries were calculated for both valsartan and benazepril by comparing the relevant peak areas of extracted samples with the peak areas of reconstituted standard. For valsartan, the recovery was calculated at 150, 2500 and 4250 ng/mL, while for benazepril, it was calculated at the nominal concentration (2.5 µg/mL) for

the low QC level.

Stability

The stability of valsartan in this validation was conducted using six QC samples for each time interval session, at both the low and the high concentration levels (150 and 4250 ng/mL).

Short-term stability

QC samples were prepared at two concentration levels and allocated for short-term stability. Six QC samples at each level were analyzed for initial concentration determination, and another six QC samples were left on the bench for 6 hours at room temperature, then analyzed. Stability was calculated by comparing the QC samples with those analyzed initially.

Freeze and thaw stability

Testing for freeze and thaw stability was determined during four freeze and thaw cycles. QC samples at low and high concentrations were prepared and stored at (-20ºC) for 24 hours and thawed unassisted at room temperature (25ºC). After complete thawing, samples were refrozen for 24 hours under the same conditions. The freeze-thaw cycle was repeated three more times; then samples were analyzed upon the fourth cycle. The samples were compared with freshly prepared samples, both calculated against standard calibration curve to obtain the results.

Long-term stability

To conduct the long-term stability, six QC samples at each level were stored at (-20ºC) for 14 days and analyzed. Stability was calculated through the comparison of the concentrations of stored samples with the freshly prepared calibration curve.

Results and Discussion

Specificity

Specificity can be evidenced due to the absence of any interfering peaks, as seen for the chromatogram's samples in figures 2, 3 and 4 for blank plasma, benazepril and valsartan respectively. Peaks for valsartan (analyte) and benazepril (IS) have different retention times, indicating the specificity of the method.

An internal standard is used during quantitative bioanalysis to correct errors in sample preparation, chromatography and detection (13). A structure analogue can be used as internal standard with key structure and functional groups same to the analyte, so that it has similar physicochemical properties (14). Therefore, benazepril was an appropriate choice for the method.

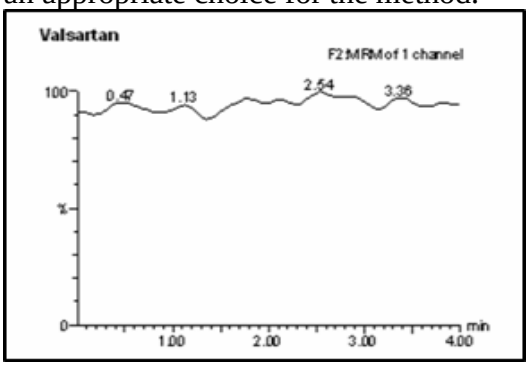

Figure (2): Chromatogram for blank

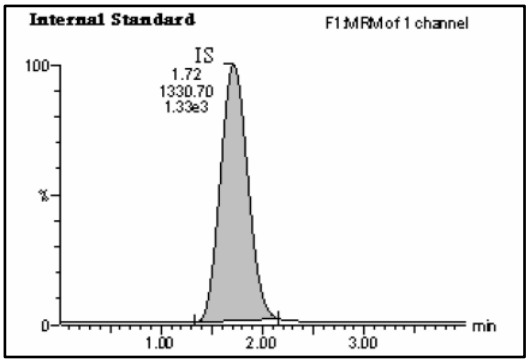

Figure (3): Chromatogram for benazepril.

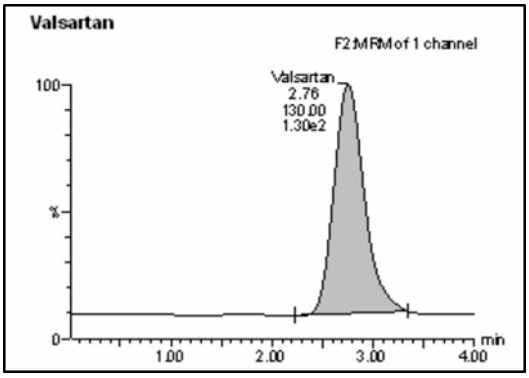

Figure 4. Chromatogram for valsartan.

An internal standard is used during quantitative bioanalysis to correct errors in sample preparation, chromatography and detection [13]. A structure analogue can be used as internal standard with key structure and functional groups same to the analyte, so that it has similar physicochemical properties [14]. Therefore, benazepril was an appropriate choice for the method.

Linearity

Method linearity is validated through constructing calibration curve, in the range from 50 to 5000 ng/mL and evaluated by weighted least squares linear regression equation. The calibration curves for each of the six days are shown in figures 5, 6, 7, 8, 9 and 10. All the regression coefficient (R2) values were not less than 0.99 which indicates an excellent relationship between the drug concentration and the response of the equipment. This is an essential part of validation to obtain test results which are directly proportional to the concentration of analyte [15]

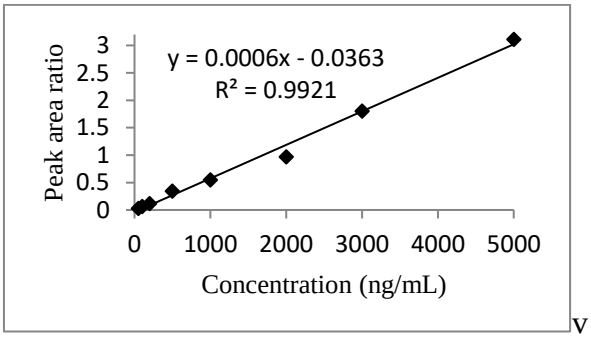

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Figure (5): Calibration curve plot of the 1st day

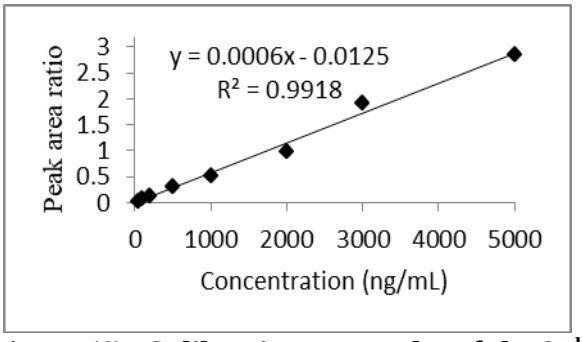

Figure (6): Calibration curve plot of the 2nd day

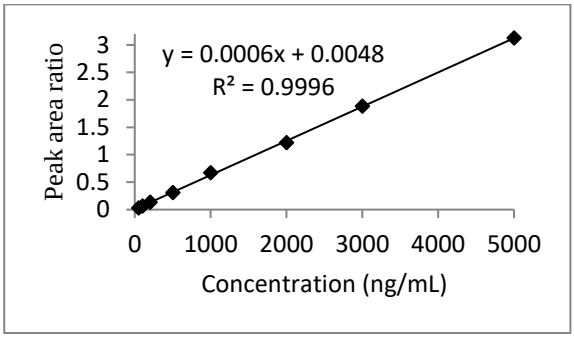

Figure (7): Calibration curve plot of the 3rd day

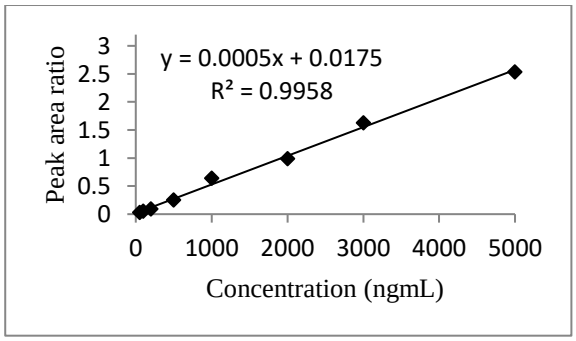

Figure (8): Calibration curve plot of the 4th day

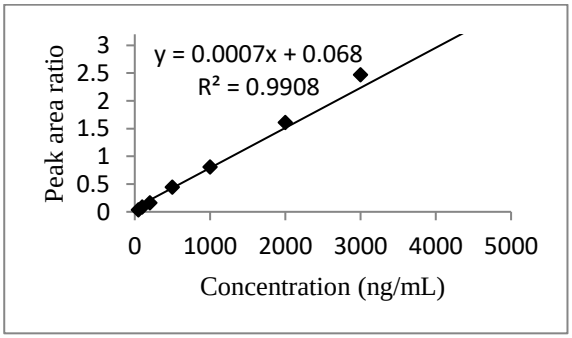

Figure (9): Calibration curve plot of the 5th day

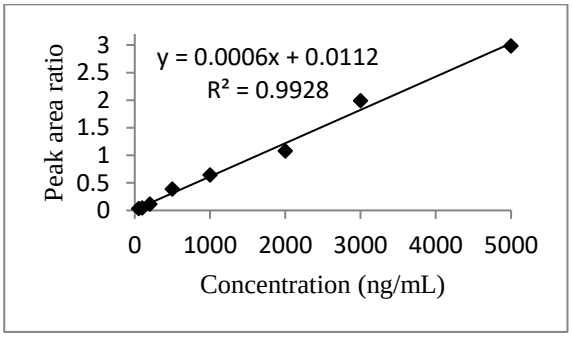

Figure (10): Calibration curve plot of the 6th day

Accuracy and Precision

Data obtained for intra and inter-day accuracy and precision of the method are presented in tables 1 and 2 respectively. Back calculated concentrations of valsartan were within 15% of the nominal values, which will ensure the method accuracy as

the closeness of agreement between the true analyte concentration and the mean result obtained by applying the same procedure to a large number of standard samples. It is related to systematic error and analyte recovery [16].

The precision of the method can also be confirmed as the coefficient of variation did not exceed 10% at all concentrations levels. It is related to the comparison of results obtained from samples prepared repeatedly under the same operating conditions over a short interval of time [17].

Nominal concentration (ng/mL)	**150**	**2500**	**4250**
Measured concentration within one day (ng/mL)	159 154 158 165 155 170 152 167 161 161 167	2166 2204 2603 2615 2609 2189 2231 2572 2238 2253 2181	4650 4322 4004 4095 4622 4380 3994 4130 4082 4003 4073
Mean	160.7	2338.	4253.7
Standard deviation	5.6	4	269.6
Precision as	3.46	194.8	6.34
	107.1	8.33	100.09

Table (1): Intra-day data of accuracy and precision of valsartan.

Nominal concentration (ng/mL)	**150**	**2500**	**4250**
Measured concentration in day 2 (ng/mL)	166 166 162 164 152 133	2435 2633 2517 2417 2576 2458	4541 4650 4137 4408 4307 4730
Measured concentration in day 3 (ng/mL)	154 141 152 155 146 131	2376 2349 2382 2245 2234 2476	4094 4044 4015 4113 4326 4316
Mean	**156.2**	**2381.6**	**4280.2**
Standard deviation	**10.2**	**167.8**	**250.6**
Precision as CV%	**6.55**	**7.05**	**5.85**
Accuracy %	**104.11**	**95.26**	**100.71**

Table (2): Inter-day data of accuracy and precision of valsartan.

Recovery

The comparison between the peak areas for the QC samples and those for pure authentic standard solutions show very good extraction recovery for valsartan and an exceptional one for benazepril.

Data representing the absolute analytical recoveries are shown in Tables 3 & 4. This parameter indicates that any analyte loss is compensated by the IS. Recovery expresses the extraction efficiency of an analytical method, as a percentage in the sample after processing steps of the method [18].

Table (3): Data of absolute analytical recovery of valsartan

No. of samples	Peak area					
	Areas of equivalent solution			Areas of QC plasma		
	150 ng/mL	2500	4250	150 ng/mL	2500	4250
1	1.1474	17.7231	33.1532	0.9750	15.0040	25.8470
2	1.0621	17.3578	33.5586	0.8879	14.8235	25.5322
3	1.1206	17.8854	33.8068	0.9633	14.7436	25.3380
4	1.1287	17.9023	33.3243	0.9767	14.1667	25.9538
5	1.1474	17.7009	32.9854	0.9222	14.1043	27.2963
6	1.1200	18.0127	33.1392	0.8327	15.6236	27.2262
Mean	1.12102	17.76370	33.32791	0.92629	14.74441	26.19891
Recovery %				82.63	83.00	78.61
Mean Recovery %				81.41		

Table (4): Data of absolute analytical recovery of internal standard (Benazepril)

No. of samples	Peak area	
	Area of equivalent	QC sample (low
1	0.30163	0.34689
2	0.29799	0.35166
3	0.29580	0.35466
4	0.30008	0.34530
5	0.30316	0.32635
6	0.30176	0.32729
Mean	**0.300070**	**0.342025**
Recovery %		**113.74**

Stability

The stability of an analyte in a given matrix under specific conditions and time intervals is a prerequisite for a reliable quantification method [19].

Short-term stability

The results of short-term stability for valsartan plasma samples at low and high levels are given in table 5. The average of six QC samples peak areas at the low and high concentration levels after 6 hours standing at room temperature did not reveal drug instability.

Freeze and thaw stability

Freezing and thawing represent one of the stress types to which an analyte in the plasma may be subjected. QC samples of valsartan at low and high concentration levels were stable when stored at -20ºC, then analyzed after 4 cycles of unassisted thawing at room temperature, as the results seen in table 6.

Long-term stability

In bioequivalence studies, plasma samples are stored frozen till the clinical part is finished, after which bio-analysis started, thus it is necessary to check stability of valsartan in the biological matrix of plasma. Stability data are given in Table 7.

Table (5): Data of short-term stability for valsartan plasma samples.

No. of samples	QC Low 150 ng/mL		QC High 4250 ng/mL	
	Initial	At 6 hours	Initial	At 6 hours
1	139	164	4275	4268
2	152	140	4450	4823
3	147	134	4581	4535
4	156	157	4481	4479
5	142	135	4653	4264
6	126	152	4442	4308
Mean	**143.7**	**147.0**	**4480.3**	**4446.2**
Standard Deviation	**10.7**	**12.5**	**130.0**	**216.2**
CV%	**7.43**	**8.47**	**2.90**	**4.88**
Accuracy %	**95.78**	**98.00**	**105.42**	**104.62**
Stability %		**102.32**	***Stability%***	**99.24**

Table (6): Data of freeze and thaw stability for valsartan plasma samples.

No. of samples	QC Low 150		QC High 4250	
	Initial	4th cycle	Initial	4th
1	140	134	4215	4410
2	128	136	4425	4393
3	138	122	4355	4239
4	130	131	4446	4303
5	119	139	4350	4498
6	136	127	4250	4184
Mean	**131.8**	**131.5**	**4340.2**	**4337.8**
SD	**7.8**	**6.22**	**92.21**	**117.1**
CV%	**5.92**	**4.73**	**2.12**	**2.30**
Accuracy %	**87.89**	**87.67**	**102.12**	**102.07**
Stability%		**99.75**	***Stability***	**99.95**

Table (7): Data of long-term stability for valsartan plasma samples

No. of samples	QC Low 150 ng/mL		QC High 4250 ng/mL	
	Initial	After 14 days	Initial	After 14 days
1	140	147	4715	4878
2	128	122	4925	4335
3	138	142	4755	4191
4	130	132	4446	4459
5	119	147	4350	4315
6	136	125	4250	4081
Mean	**131.8**	**135.8**	**4573.5**	**4493.2**
SD	**7.8**	**11.2**	**263.6**	**275.7**
CV%	**5.92**	**8.27**	**5.76**	**6.14**
Accuracy	**87.89**	**90.56**	**107.61**	**105.72**
Stability%		**103.03**	***Stability%***	**98.24**

Conclusions

A developed method approved to be sensitive and specific assay for valsartan. It was shown that this method is applicable for the analysis of valsartan in human plasma samples during bioequivalence study. The sample preparation method applied protein precipitation and solvent extraction for sample purification, without solid-phase extraction (SPE). In addition, only single mass spectroscopy (MS) was applied in this method (not MS/MS) (20). Therefore, this method is more applicable and feasible, compared to the other more sophisticated methods. Samples collection, handling, processing and running should take into consideration the stability conditions furnished by the stability tests in this validation study. A standard calibration curve should be generated in each analytical run-in order to be used for the determination the concentrations in the unknown samples. Furthermore, QC samples at low, medium and high concentrations should be injected. QC samples should be analyzed together with the unknown samples in order to detect any analytical drift.

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