Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 100–105

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantitation of irbesartan and major proteins in human plasma by mass spectrometry with time-of-flight analyzer

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ARTICLE INFO

Article history:
Received 8 April 2010
Received in revised form 13 June 2010
Accepted 19 July 2010
Available online 19 August 2010

Keywords:
MALDI-TOF
Irbesartan
Angiotensin II receptor blocker
Chemical analogues
Nanoliquid chromatography

ABSTRACT

A simple matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method was developed to analyze irbesartan in human plasma. Irbesartan is a kind of angiotensin II receptor blocker (ARB) and is used as an antihypertensive drug. MALDI-TOF MS is a rare application for clinical drug analysis in human plasma. After simple micro-liquid–liquid extraction, irbesartan-containing supernatant was spotted on a target plate, mixed with matrix and then detected by MALDI-TOF MS within the clinically therapeutic range. Furthermore, we used cheaper chemical analogues to label the major proteins in human plasma for protein quantitation. After enzyme digestion, peptide mixtures were injected into nanoliquid chromatography (nanoLC) coupled with tandem mass spectrometry (MS–MS). Protein identification could be carried out simultaneously by peptide sequencing and database searching. Chemical analogue labeling method is an alternative way for expensive isotope reagents. Quantity change of proteins before and after administration of irbesartan could be detected by this method. Application of these methods in human plasma demonstrated that these two micro-scale MS methods used for clinical drug monitoring, protein quantitation and identification are successful.

1. Introduction

Irbesartan, an angiotensin II receptor blocker (ARB), is widely used for the treatment of hypertension and heart failure in clinical patients. Angiotensin II is an octapeptide regarded as the main effector of AT1 receptor in renin–angiotensin system. It causes vasoconstriction, tachycardia, increase of aldosterone secretion from the adrenal cortex and retention of sodium and body fluid [1]. These compounds are the key factors in raising blood pressure. Irbesartan (Aprovel® or Avapro®) is a selective non-peptidic angiotensin AT1 receptor antagonist associated with a lower incidence of side effects than other classes of antihypertensive drugs. Irbesartan also demonstrated superior antihypertensive efficacy versus losartan and valsartan [2]. ARBs have been the choice of drugs in diabetic nephropathy by World Health Organization (WHO)/International Society of Hypertension (ISH) guidelines [3].

Several analytical methods have been developed for the determination of irbesartan including liquid chromatography (LC) [4–15], capillary electrophoresis (CE) [16–19] and spectrophotometry [20–22]. LC is the major method for measurement of irbesartan in human plasma and urine. It is combined with ultraviolet (UV) detector [4,5], diode array detector (DAD) [6–8], fluorescence (Flu) detector [9–13], electrospray ionization mass spectrometric detection [14], and tandem quadrupole mass spectrometer [15]. In the CE technique, all the relative references apply in pharmaceutical formulation, except Zhang et al. used polymer monolith microextraction as a clean-up procedure and capillary zone electrophoresis to analyze irbesartan and three other sartans in urine [16]. The spectrophotometric methods are all used in pharmaceuticals.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is widely used in high molecular weight compound (such as proteins or polymers) analysis and is almost nearly a solvent-free green method. MALDI-TOF MS is a high throughput technique but seldom used in low molecular weight compound analysis. In all documented references, no MALDI-TOF MS method has been used to determinate irbesartan presence and concentration in human plasma until now. When drug analysis is conducted on biosamples, the pretreatment step is necessary to exclude the undesired compounds. There are many sample preparation methods used in biological samples, such as solid-phase extraction (SPE) [6–8,10,11,14], solid-phase microextraction (SPME) [9], liquid–liquid extraction (LLE) [12,15] and protein precipitation [4,5,13]. SPE is the principal way to clean-up the biosamples, but it is complicated and time-consuming. SPME is a very suitable sample preparation technique for a small amount of samples, but it is not widely used in human plasma samples. Protein

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doi:10.1016/j.jpba.2010.07.019

precipitation is an easy method, but this strategy is not suitable for MALDI-TOF MS because of high salty biosample will interfere with the ionization of desired analytes. LLE is an economic and simple pretreatment method, but the structure of irbesarten causes difficulty in extraction into an organic solvent. The sample solution needs to be acidified to transform irbesartan from salt form into free form in biological fluids.

For protein analysis, LC separation technique coupled with tandem mass spectrometry (MS–MS) is a powerful platform for protein qualitation and quantitation [23,24]. Protein identification is based on the enzyme digestion and database searching. After digestion, proteins are cleaved into peptides and peptide ions are fragmented by collision-induced dissociation (CID). Fragment ions from CID could be matched with the fragment patterns in the database, and then proteins could be identified according to comparison of the theoretical ion templates with the experimental ion profiles. Protein quantitation is usually carried out by isotope labeling strategy, such as isotope-coded affinity tags (ICAT) [25,26], isobaric tag for relative and absolute quantitation (iTRAQ) [27,28] and stable isotope labeling with amino acids in cell culture (SILAC) [29,30]. The major demerit of isotope labeling is the high cost of the isotope reagent. Chemical analogues are usually used as an alternative way for quantitative purpose when isotope internal is unavailable.

In this study, a rapid MALDI-TOF method is investigated to determinate the irbesartan concentration in human plasma. In our approach, we adjusted the sample solution to acidic environment, and then we used only 10 μL organic solvent to extract irbesartan and clean-up at the same time. Furthermore, we used a cheaper derivatization method coupled with LC–MS–MS to compare the plasma protein quantity change before and after administration of irbesartan. We used cheaper chemical analogues as labeling reagents to replace the expensive isotope to analogues as labeling reagents. Derivatization of proteins by Michael addition reaction is used to evaluate the protein expression before and after medication with irbesartan. This is the first time to determine irbesartan and major proteins in human plasma by MS. The whole study will provide a quantitative strategy for clinical drug monitoring and major protein identification in human plasma.

2. Materials and methods

2.1. Reagents

Irbesartan and losartan potassium (internal standard, IS) were purchased from U.S. Pharmacopeia (Maryland, USA). Trifluoroacetic acid (TFA), alpha-cyano-4-hydroxycinnamic acid (CHCA), methyl vinyl ketone (MVK), ethyl vinyl ketone (EVK) and dithiothreitol (DTT) were purchased from Sigma–Aldrich (St. Louis, MO). Formic acid (FA), n-hexane, ethyl acetate, toluene, methanol, acetonitrile (CH_3CN) and ammonium bicarbonate (NH_4HCO_3) obtained from Merck (Darmstadt, Germany) were chromatographic grade. Milli-Q water purification system was manufactured by Millipore (Bedford, MA, USA) and used to prepare the aqueous solutions. Trypsin (modified for protein sequencing) was obtained from Promega (Madison, WI, USA). The dealbumin kit "ProteoSpin" was purchased from Norgen Biotek Corporation (Thorold, ON, Canada).

2.2. Preparation of working solutions

Irbesartan and losartan stock solution (1 mg/mL) were prepared by dissolving the appropriate amounts of these chemicals in methanol and water, respectively. FA and NH_4HCO_3 aqueous solution were prepared by adding these compounds to water to give a concentration of 5% and 25 mM, respectively. The DTT, MVK and EVK solutions were prepared by adding appropriate amounts of these compounds to 25 mM NH_4HCO_3, the final concentrations of them were 25 mM. Trypsin solution (20 ng/μL) was also prepared by 25 mM NH_4HCO_3.

2.3. Instrumentation

A MALDI-TOF MS system, model Autoflex III Smartbeam, equipped with a nitrogen laser radiating at 355 nm from Bruker Daltonics (Billerica, MA, USA) was used to obtain the data in positive ion reflector mode. The nanoLC system was comprised of three pumps (pump A, B and C), an autosampler, an on-line degasser, a sample cooler, a syringe pump and a switching valve. The separation was performed on a Micro-tech Scientific Inc. (Vista, CA, USA) 10 cm reversed-phase C18 nano-flow column (150 μm inner diameter; 375 μm outer diameter; 3 μm particle size). A desalting column (C18 PepMap, 300 μm ID, 5 mm) used for peptide enrichment and trapping was purchased from LC Packings (Sunnyvale, USA). The mass spectrometer was a quadrupole time-of-flight (Q-TOF) mass spectrometer (model Global Ultima) from Waters-Micromass (Milford, MA, USA) interfaced with a nanospray source (20×90 μm fused-silica capillary).

2.4. MALDI-TOF and nanoLC-ESI-Q-TOF conditions

For irbesartan analysis by MALDI-TOF, 1 μL of the sample solution extracted from plasma was spotted on a target plate (Bruker Daltonics) then 1 μL of matrix (CHCA) was added. CHCA (10 mg/mL) solution was dissolved in CH_3CN: 0.1% TFA = 50:50 (v/v). Mass spectra were collected for the summing of 2000 laser shots. Data processing was performed by FlexAnalysis software (Bruker Daltonics).

For protein identification and quantitation by nanoLC-ESI-Q-TOF, peptides from enzyme digestion were enriched and desalted by a small trapping column. For on-line desalting, 0.1% FA with a flow rate of 30 μL/min was carried out by pump A. After washing out the undesired compounds, peptides were fractionated and separated on a nanoLC column for 240 min (flow rate = 400 nL/min). Mobile phase B and C for peptide analysis from pump B and C were 0.1% FA:CH_3CN = 95:5 (v/v) and 0.1% FA:CH_3CN = 5:95 (v/v), respectively. The initial nanoLC condition was B–C 90:10 (v/v) at 0–3 min. Then the mobile phase composition was linearly changed to B–C 25:75 (v/v) at 3–180 min. Finally, the gradient condition was linearly changed to B–C 90:10 (v/v) at 180–240 min.

For MS system, the nanosource voltage was set at 3.2 kV and temperature was set at 80 °C. The cone voltage was worked at 80 V in positive ion mode and the TOF analyzer was operated at V-mode for peptide analysis. The MS and MS–MS spectra were acquired in a smart data-dependent acquisition mode and argon was used as the collision gas. MassLynx 4.0 software was used to process the precursor and fragment ions data. Then these spectra were converted to the peak lists in pkl file format. For protein identification, these pkl files were submitted to an in-house MASCOT search engine. The parameters of MASCOT were set as follows: database, NCBInr; taxonomy, Homo sapiens; enzyme, trypsin; variable modifications, oxidation (M), MVK (C, alpha-NH_2) and EVK (C, alpha-NH_2); peptide and MS–MS ion mass tolerance, 0.3 Da; peptide charge, 2+ and 3+; data format, pkl; instrument, ESI-QUAD-TOF.

2.5. Extraction of irbesartan from human plasma for MALDI-TOF analysis

Sample preparation for irbesartan analysis by MALDI-TOF analysis was simple and fast. Briefly, drug-free human plasma samples were spiked with five different levels of irbesartan solution to prepare the final concentrations in human plasma over the range of

0.1–10 μg/mL. Human plasma samples (9 μL) were added in Eppendorf vials and then IS solution (1 μL; 600 μg/mL) was added. The sample vials were vortexed (30 s) and 5% FA (10 μL) was added. Then 10 μL of toluene was added for irbesartan extraction. Sample vials were well mixed for 30 s and centrifuged for 2 min (at 10,000 rpm). After centrifugation step, 1 μL of the analytes containing supernatant was spotted on a target plate (anchor chip) followed by addition of 1 μL CHCA. Then the target plate was subjected to MALDI-TOF for irbesartan analysis.

2.6. Major protein preparation for nanoLC-ESI-Q-TOF analysis

Before and after medication, a single dose of 150 mg irbesartan tablet (Aprovel®), plasma samples were collected. Plasma dealbumin procedures were adapted by the manufacturer protocol. Simply speaking, the dealbumin column was activated. Then 10 μL plasma was mixed with 490 μL buffer and loaded on the activated column. After the washing step, the albumin was depleted and this albumin rich solution was collected. Next step, the dealbumin protein solution was eluted, collected and neutralized. This dealbumin protein mixture was called dealbumin solution. For protein enrichment, 100 μL albumin rich and dealbumin solution were transferred into Eppendorf then mixed with acetone (1000 μL), vortexed (30 s) and centrifuged for 10 min (at 10,000 rpm). After precipitation and centrifugation, the supernatant was thrown away and the protein residues were collected and dried completely. To re-dissolve the protein sediments, 100 μL of 25 mM NH_4HCO_3 aqueous was added and well mixed. Afterward 16 μL of these protein mixtures and 2 μL of DTT aqueous were pipetted into Eppendorf and placed at 25 °C for 30 min for protein reduction.

After reduction the disulfide bonds of proteins, 2 μL of 25 mM MVK (for control sample) and EVK (for experimental sample) solution was added for quantitation labeling and kept at 25 °C in the dark for 30 min. In this study, control and experimental mean plasma samples before and after medication with 150 mg irbesartan were analyzed. For protein digestion, 2.5 μL trypsin solution was added into the protein mixtures and placed at 37 °C for 16 h. After overnight digestion, control and experimental samples were mixed in 1:1 ratio then 5 μL of this peptide mixture solution was injected into the nanoLC-ESI-Q-TOF system.

3. Results and discussion

The chemical structure of irbesartan contains a tetrazole ring; the pK_a [31] of irbesartan is approximately 5 and will be ionized at normal physiologic pH. Acidification of the plasma is necessary in order to suppress the ionization of irbesartan in the liquid–liquid extraction step. Isotope-labeled irbesartan is absent, so we used the structural analog losartan as the IS for quantitation purpose. The structures of irbesartan and losartan are shown in Fig. 1.

3.1. Quantitation of irbesartan by MALDI-TOF

In order to extract sufficient irbesartan from plasma for clinical monitoring, the effects of the organic acid and extraction solvent on the extraction of the irbesartan were evaluated and optimized. The concentration of irbesartan used for the study was 10 μg/mL. The effects of different percentages (0–20%) of the FA on the extraction efficiency of irbesartan were studied. The results in Fig. 2 indicate that the extraction efficiency (%) is attained at the plateau when the concentration of FA is over 5%. The FA at 5% was used for the extraction of irbesartan from human plasma.

In this study, micro-liquid–liquid extraction strategy was used to isolate irbesartan from human plasma. We tried to directly detect irbesartan from plasma without extraction procedure, no

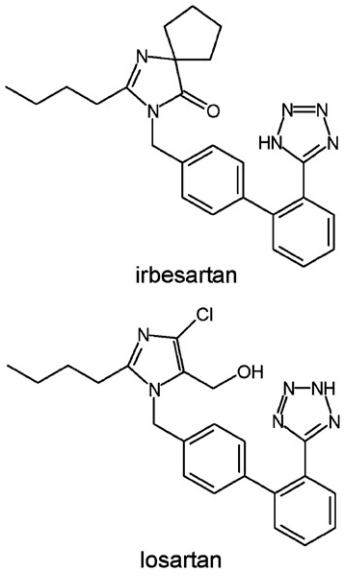

Fig. 1. Chemical structures of irbesartan and losartan (IS).

irbesartan signal could be detected by MALDI-TOF MS. Water-immiscible solvents (such as n-hexane, ethyl acetate and toluene) were tested to evaluate the extraction efficiency. Liquid–liquid extraction is a simple way for sample enrichment and purification from complicated matrixes. Organic solvents contain different physical–chemical properties to purify and concentrate irbesartan from human plasma to organic layer. The extraction efficiency (%) of n-hexane, ethyl acetate and toluene for extraction the initial amount of irbesartan are 0, 16.5 and 97.3%, respectively. Because of the different partition coefficients of irbesartan in different immiscible solutions, this compound is distributed easily from plasma to toluene layer. Fig. 3 shows that toluene is the best water-immiscible solvent to retain irbesartan in the organic layer. The $[M+H]^+$ of Los (losartan; IS) and Irb (irbesartan) are m/z 423 and m/z 429.The MALDI-TOF mass spectra of irbesartan isolated from human plasma are shown in Fig. 4A. The mass spectra from the plasma samples show that no matrix peaks interfere with the Los and Irb in the analysis.

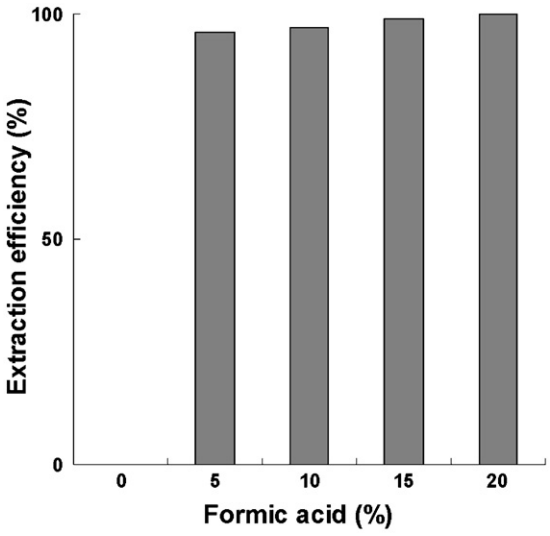

Fig. 2. Effect of the percentages of formic acid (FA) on the extraction of irbesartan from human plasma.

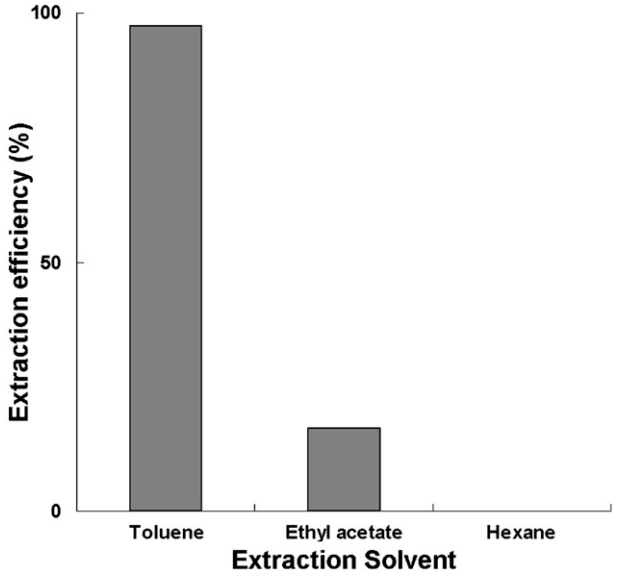

Fig. 3. Effect of the organic solvents on the extraction of irbesartan from human plasma.

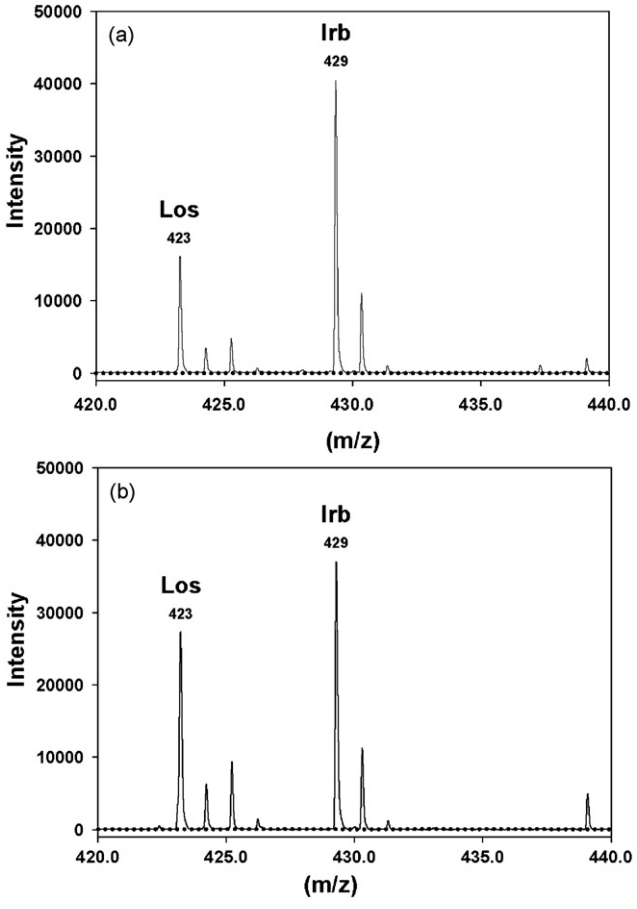

Fig. 4. MALDI-TOF mass spectra of plasma samples: (A) plasma spiked with irbesartan (5 μg/mL) and losartan (600 μg/mL); (B) plasma sample of a healthy volunteer after medication with an oral dose of 150 mg irbesartan tablet. The $[M+H]^+$ of Los (losartan; IS) and Irb (irbesartan) are *m*/*z* 423 and *m*/*z* 429. Dotted lines in (A) and (B) are plasma blank and plasma before dosing irbesartan tablet.

Table 1
Precision and accuracy for the determination of irbesartan spiked in human plasma by MALDI-TOF.

Concentration known (μg/mL)	Concentration found (μg/mL)	R.S.D. (%)	R.E.[c] (%)
Intra-day[a] ($n=6$)			
0.40	0.42 ± 0.05	11.9	+5.0
0.80	0.73 ± 0.06	8.2	−8.8
2.00	2.02 ± 0.04	2.0	+1.0
5.00	4.80 ± 0.29	6.0	−4.0
Inter-day[b] ($n=6$)			
0.40	0.39 ± 0.04	10.3	−2.5
0.80	0.73 ± 0.05	6.9	−8.8
2.00	2.14 ± 0.11	5.1	+7.0
5.00	5.04 ± 0.13	2.6	+0.8

[a] Intra-day assay variance from analysis of irbesartan at six intervals on a single day.
[b] Inter-day assay variance from analysis of irbesartan on 6 consecutive days.
[c] R.E. calculated from (value found − value known)/value known.

Quantitation of irbesartan was carried out by integrating the peak area of irbesartan (*m*/*z* 429) and IS (*m*/*z* 423) for different concentrations. The calibration curve for the analysis of irbesartan in spiked plasma for a series of levels ranging from 0.1 to 10 μg/mL was constructed. The linearity was calculated by the peak area ratio (peak area of irbesartan/peak area of IS) as the *y*-axis and the concentration (μg/mL) of the irbesartan as the *x*-axis. The linear equation for the concentration versus the ratio of peak area ratio was $y = (0.5948 \pm 0.0648)x - (0.0675 \pm 0.0513)$ with a correlation coefficient over 0.995 ($n = 6$).

The results showed that good linearity was obtained for the analysis of irbesartan in human plasma. The limit of quantification (LOQ) and the limit of detection (LOD) of irbesartan were 50 and 25 ng/mL, respectively. Compared with other methods [4,5,7,9,10–15], the LOD and LOQ of these methods were 1–980 and 3–2980 ng/mL. Plasma (or serum) samples of these methods were over 100 μL (100–1000 μL), only 10 μL plasma was used in our method. For clinical test, a small amount of plasma sample (10 μL) is friendly and acceptable for patients. The relative standard deviation (R.S.D.) and relative error (R.E.) for the analysis of irbesartan are evaluated at four levels, 0.4, 0.8, 2 and 5 μg/mL. The precision and accuracy estimated by R.S.D. and R.E. are shown in Table 1. Table 1 indicates that the R.S.D. and R.E. values for the intra- and inter-day analyses of irbesartan spiked in plasma are all below 12 and 9%, respectively. For stability tested, plasma samples are stored at −20 °C and the stability of irbesartan were studied over a period of 7 days. No significant change of irbesartan signal was found, indicating that irbesartan is sufficiently stable before MALDI method analysis. This MALDI-TOF method was further applied to analyze the irbesartan plasma level in a healthy volunteer. Human plasma sample was prepared before and after oral medication of a single dose of 150 mg irbesartan tablet (Aprovel®). The peak plasma concentration was 2.62 ± 0.14 μg/mL ($n = 3$) after oral a single dose for 2 h. The MALDI-TOF mass spectra of plasma samples of a healthy volunteer before and after medication are shown in Fig. 4B. These results indicate that the sensitivity of this MALDI-TOF method is sufficient for micro-scale monitoring of irbesartan in 10 μL human plasma.

3.2. Quantitation of major proteins by nanoLC-ESI-Q-TOF

MVK and EVK are chemical analogues and they could react with the -SH of cysteine or alpha-NH_2 of the proteins by Michael addition. After chemical labeling, modified trypsin was added in order to break down proteins then peptides were identified by LC–MS–MS. For protein quantitation, we added four new variable modifications in the MASCOT: MVK (in cysteine or alpha-NH_2) and EVK (in cys-

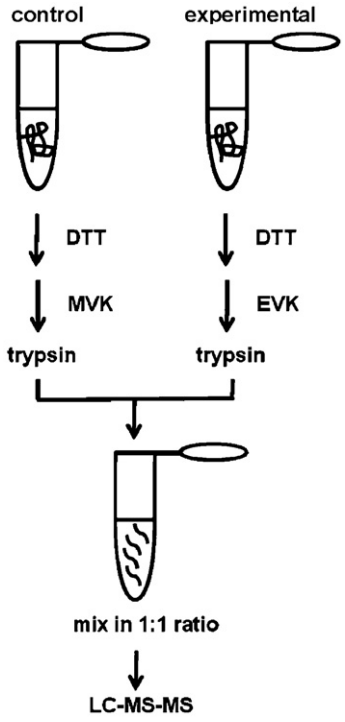

Fig. 5. The chemical analog labeling for protein quantitation and identification. In this study, control and experimental samples mean plasma before and after administrating with a tablet containing 150 mg irbesartan.

teine or alpha-NH_2). Theoretically, the mass difference (ΔM) of the MVK and EVK pair in the same peptide is 14, 7 and 4.7 Da for single (+1), double (+2) and triple (+3) charges, respectively. The detailed flowchart for protein quantitation and identification is shown in Fig. 5. Briefly, the disulfide bonds of proteins were reduction by adding DTT via the thiol-disulfide exchange reaction. Then proteins were reacted with MVK and EVK in order to form the quantitative tags. Most peptides containing double (+2) and triple (+3) charges are easily observed in the nanoESI-Q-TOF system because the cutting sites of trypsin are lysine and arginine after digestion. Control and experimental samples were mixed in 1:1 ratio and peptide mixtures were analyzed and identified by nanoLC-ESI-Q-TOF system coupled with database searching.

The relative peak area ratio of the precursor ions labeled with MVK and EVK were used for quantitation; the fragment ions of the precursor ions labeled with MVK and EVK were used for identification. For instance, the precursor spectra for quantitation and sequence for identification are shown in Fig. 6. Fig. 6A shows that the sequence VDGALCMEK from the protein hemopexin precursor (gi|386789) was labeled with MVK and EVK individually. The ratio of the sequence VDGALCMEK labeled with MVK and EVK was 1. The results indicate that hemopexin precursor was equal before and after medication. Fig. 6B shows that the sequence ADRDQYELLCLDNTR from the protein transferrin (gi|37747855) was labeled with MVK and EVK in the cysteine. The ratio of the sequence ADRDQYELLCLDNTR labeled with MVK and EVK was 0.25. The results indicate that transferrin is 4-fold up-regulated after medication.

There are over 100 major proteins identified by this strategy. Only a small amount of proteins were up- or down-regulated and the results are shown in Table 2. The results indicate that the amount of immunoglobulin heavy chain (gi|10334611), lipid-free human apolipoprotein A-I (gi|90108664), apolipoprotein A-II preproprotein (gi|4502149), immunoglobulin heavy chain VDJC region (gi|553426) and immunoglobulin light chain (gi|149673887) are up-regulated, while Ig kappa chain V-I region AG (gi|125756), lipoprotein Gln I (gi|229479) and transferrin (gi|37747855) are down-regulated before and after medication of irbesartan. According to the data from Table 2, this chemical labeling procedure is proved workable in biological samples. The sensitivity of this

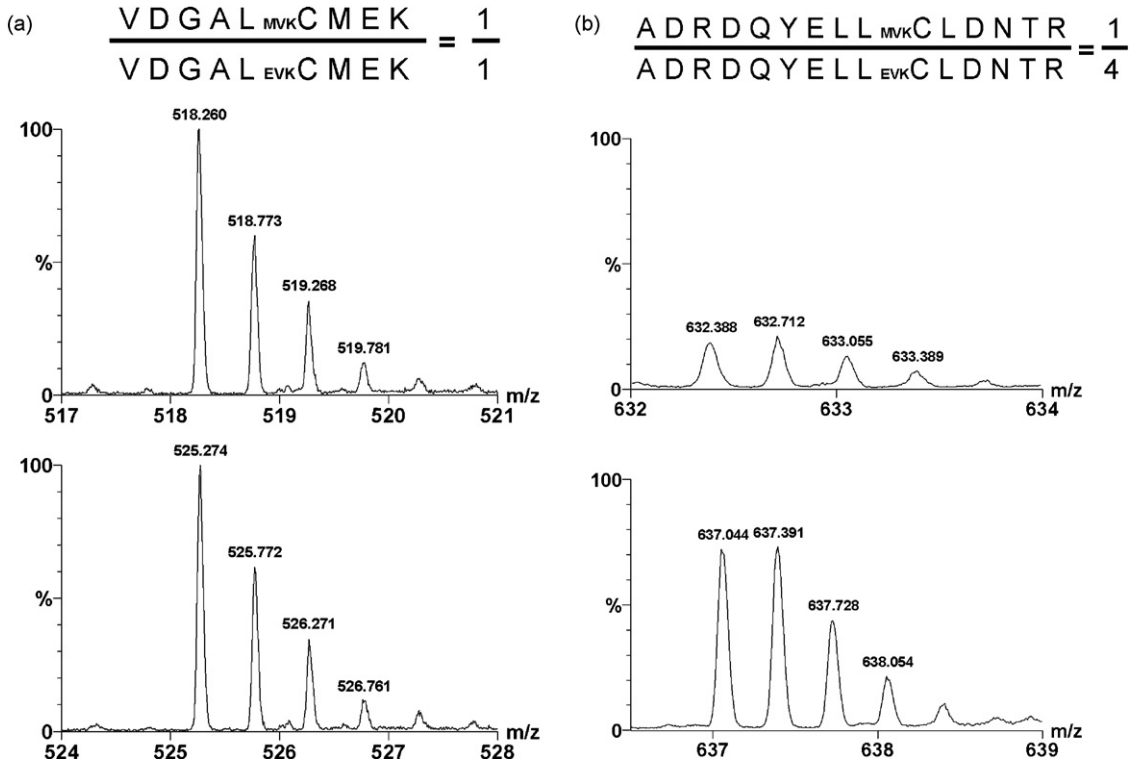

Fig. 6. The precursor spectra for quantitation and peptide sequence for identification. (A) Hemopexin precursor was equal and (B) transferrin was 4-fold up-regulated.

Table 2
Protein quantitation and identification by nanoLC-ESI-Q-TOF before and after medication of a single dose of irbesartan.

Accession no.	Protein name	Fold (MVK/EVK)[a]
gi|10334611	Immunoglobulin heavy chain	12.15
gi|90108664	Lipid-free human apolipoprotein A-I	6.05
gi|4502149	Apolipoprotein A-II preproprotein	3.72
gi|553426	Immunoglobulin heavy chain VDJC region	2.57
gi|149673887	Immunoglobulin light chain	1.75
gi|125756	Ig kappa chain V-I region AG	0.62
gi|229479	Lipoprotein Gln I	0.35
gi|37747855	Transferrin	0.25

[a] Peptide labeled with MVK and EVK refer to proteins before and after medication with irbesartan.

micro-scale method is sufficient for identification and quantitation the major proteins in human plasma. For protein quantitation, these chemical analogues are cheaper than isotope reagents.

4. Conclusion

This study provides two micro-scale MS methods; the MALDI-TOF and nanoLC-ESI-Q-TOF methods, for clinical drug monitoring, protein quantitation and identification. These methods can reduce the production of organic solvent waste and only micro-liter levels of plasma are sufficient for clinical testing. The sensitivity of the MALDI-TOF method is adequate for irbesartan monitoring in the clinical therapeutic range. Non-isotopic analogues, MVK and EVK, can also successfully react with proteins for quantitation and identification by nanoLC-ESI-Q-TOF before and after medication of a single dose of irbesartan.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the National Science Council (NSC 97-2113-M-037-008-MY2 and NSC 98-2113-M-037-010), Kaohsiung Medical University (Q-098002 and Q-098004) and Center of Excellence for Environmental Medicine (Kaohsiung Medical University) for financial support of this work. The authors are also grateful to the Center for Resources, Research and Development (Kaohsiung Medical University) for instrumental support of this work.

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