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Rapid and Sensitive Analysis of Valproic Acid in Human Red Blood Cell by LC-MS/MS

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A sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was developed to determine valproic acid in human red blood cell (RBC). It is important to measure the drug concentration of the RBC as well as that of the plasma because of drug partitioning for pharmacokinetic and pharmacodynamic study. The method was linear over the dynamic range of 1-100 μg/mL with a correlation coefficient $r = 0.9997$. The linearity of this method was established from 1 to 100 μg/mL for valproic acid in red blood cell with accuracy and precision within 15% at all concentrations. The intra-run and inter-run assay accuracy and coefficient of variations are all within 15% for all QC samples prepared in plasma and red blood human samples. Then, valproic acid amount by protein precipitation in plasma was quantified by LC-MS/MS mass spectrometry. The distribution ratio of VPA in RBC and plasma was analyzed by clinical samples. Based on measurement of the valproic acid in human red blood cell, this method has been applied to clinical research for study of distribution ratio of valproic acid in blood.

Key Words : Valproic acid, LC-MS/MS, Human red blood cell, Validation, RBC partitioning

Introduction

Valproic acid (2-propyl pentanoic acid, VPA; Figure 1(a)) is a broad-spectrum antiepileptic drug with unique anti-convulsant properties and is used in the treatment of primary generalized seizures, partial seizures and myoclonic seizures.[1,2] Valproic acid sodium salt (sodium 2-propylpentanoate) dissociates to the valproic acid ion in the gastrointestinal tract and exists as valproic acid ion in the blood.[3] It is necessary to optimize these parameters carefully to achieve a sensitive and specific method for the analyses of VPA concentration in blood. Many analytical method is reported in scientific literature for the quantification of VPA in biological matrices.[4,5] The early days, VPA is analyzed by gas chromatography (GC) with flame ionization detector[6,7] or by an immunological assay.[8] GC analysis is difficult that it is required to extract the drug with an organic solvent and immunological techniques are expensive. Various bioanalytical methods of VPA which included high-performance liquid chromatography (HPLC)[9] had been developed, but it consumed significant analysis time and large amounts of samples. In recent years, simple, selective and highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) were developed and validated. As above, several HPLC and LC-MS/MS methods have been developed for the analysis of VPA in plasma. However, no study has demonstrated LC-MS/MS method for the VPA in red blood cells. S. Yu *et al*. reported a method for measuring compound partitioning between human plasma and red blood cells (RBC) in a drug discovery field with LC-MS/MS-based depletion assay.[10] The information on the partitioning of a compound of pharmaceutical interest in RBC is important in understanding the pharmacokinetics and pharmacodynamics of the compound and is sometimes critical in screening candidates of a drug without a lost opportunity.

The aim of this research was to explore the quantification of VPA in human RBC using benzoic acid (Fig. 1(b)) as the internal standard (IS).[11] The high selectivity of a triple quadrupole MS system with an electrospray ionization (ESI) interface used for the development and validation with a reversed-phase LC-MS/MS method in multiple reaction monitoring (MRM) mode. MRM, using the respective $[M-H]^-$ ions, is utilized for detection monitoring at *m/z* 143.2-143.2 for valproic acid and *m/z* 121.1-77.1 for benzoic acid. The method has been validated and has been applied to the pharmacokinetics and pharmacodynamics study of distribution ratio of valproic acid in human red blood cell and plasma.

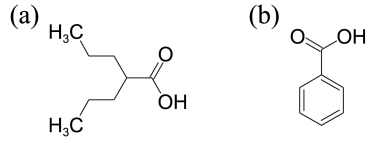

Figure 1. Chemical structures of (a) valproic acid and (b) benzoic acid.

[a]These authors contributed equally to this work.

Experimental

Chemicals and Reagents. Valproic acid (Sodium valproate) and benzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical and HPLC-grade. The water, methanol, acetonitrile and ethyl ether used for LC-MS/MS were purchased from Fisher Scientific (Korea). The ethyl acetate, *tert*-butyl methyl ether and dichloromethane used as extraction organic solvent were purchased from Duksan (Korea), Daejung (Korea) and J.T. Baker Co (NJ, USA) respectively. The acetic acid was purchased from Junsei (Japan).

Sampling and Preparation. The human whole blood samples were gently mixed by inverting the tube 4 to 6 times and immediately stored at −70 °C for cell lysis. After the cell lysis, the content of each sample was directly used for sample extraction. The extraction efficiency of VPA and IS from RBC in human was examined using protein precipitation using methanol, acetonitrile and liquid-liquid extraction using *tert*-butyl methyl ether, ethyl acetate and dichloromethane as previously described.[12]

50 μL of RBC was diluted with 50 μL of water and the diluted RBC was then spiked with 10 μL each of 1000 μg/mL VPA and 100 μg/mL IS. For the extraction by protein precipitation, 250 μL of methanol or acetonitrile was added and the mixture was vortex-mixed at high speed for 2 min. After centrifugation for 6 min at 13,000 rpm and 4 °C (Thermo, Germany), the supernatant was transferred into a HPLC vial and 10 μL of the sample was loaded into the LC-MS/MS for analysis.

For the extraction by liquid-liquid extraction, 50 μL of water was added to 50 μL of RBC and the diluted RBC was spiked with 10 μL each of 1000 μg/mL VPA and 100 μg/mL IS. Each 1250 μL of *tert*-butyl methyl ether, ethyl acetate or dichloromethane was then added and the mixture was vortex-mixed at high speed for 3 min. The mixture was centrifuged for 15 min at 13,000 rpm and 4 °C. The 1190 μL of the supernatant was transferred into a clean eppendorf tube and evaporated to dryness for 30 min at 30 °C under evaporation (Eppendorf AG, 22331 Hamburg, Germany). The residue was reconstituted with 100 μL of 40% acetonitrile in water, vortex-mixed for 2 min, sonicated for 5 min and then centrifuged for 10 min 13,000 rpm and 4 °C, 50 μL of supernatant was transferred into a HPLC vial and 10 μL was injected for LC-MS/MS analysis.

For the analysis of VPA in human plasma, the protein precipitation method was applied. The extraction process was according to above described method.

Chromatography and Mass Spectrometry. LC-MS/MS method development and validation were performed using an Agilent 1100 HPLC system (Agilent, Applied Biosystems) interfaced with a triple quadruple mass spectrometer equipped with Turbo Ion Spray interface (4000QTRAP, Applied Biosystems, Foster City, CA, USA) MS data acquisition and processing were performed using the Analyst Software v1.4.2 (Applied Biosystems). All MS experiments were performed using eletrospray negative ionization mode.

According to development, tuning of MS parameters in both positive and negative ionization modes was carried out for valproic acid and benzoic acid (IS). However, the response found was much higher in negative ionization mode for valproic acid compared to that in positive mode due to its acidic nature. Thus, MS experiments were performed using eletrospray negative ionization mode. For MS optimization, 1 μg/mL each of VPA and IS in methanol were infused separately into the QTRAP MS at a flow rate of 10 μL/min using a built-in microsyringe pump (Harvard) Apparatus Inc, Holliston, MA, USA) for the optimization of the compound-dependent MS parameters (declustering potential (DP), enterance potential (EP), collision energy (CE) and collision cell exit potential (CXP) using multiple reaction monitoring (MRM) experiment. The collision-activated dissociation (CAD) gas was set at 'Medium' throughout all experiments. The optimized compound-dependent parameters of VPA and IS were used for all subsequent experiments. 10 μL of 500 ng/mL of VPA in methanol:water (50:50) was introduced into the LC-MS/MS system by compound optimization of the source-dependent MS parameters (temperature (TEM), nebulizer gas 1 (GS1), nebulizer gas 2 (GS2), ionspray voltage (ISV) and curtain gas (CUR)) for each mobile phase combination at a flow rate 1 mL/min were used (Table 1).

In order to optimize the mobile phase, triplicate LC-MS/MS analyses of mixture containing 500 ng/mL of VPA and 500 ng/mL of IS in methanol:water (50:50) were performed consecutively using each of the investigated mobile phase combination. All chromatographic separations were performed using a BetaBasic-8 (C18, 4.6 × 100 mm i.d, 5 μm particle size) (Thermo) at a column temperature of 25 °C and mobile phase flow rate of 1 mL/min, 0.1% acetic acid (pH 4.0)/methanol was most optimum in terms of sensitivity and peak shape of VPA and the benzoic acid. The peak width signal-to-noise (S/N) ratio and integrated peak areas of VPA and IS were documented for each of the mobile phase combinations.

Method Validation. Stock solutions of VPA were prepared in methanol at a free base concentration of 1 mg/mL. Secondary and working standard solution was prepared from stock solutions by dilution with methanol. These diluted

Table 1. Ion source and analyte-dependent MS parameters

Source parameter	Value
Curtain gas, psi	20
Ionspray voltage, V	-450
Temperature, °C	350
GS 1, psi	50
GS 2, psi	50
EP, V	-10
CXP, V	-10
DP for VPA, V	-114
DP for BA, V	-47.1
CE for VPA, V	-10
CE for BA, V	-16.3

working standard solutions were used to prepare the calibration curve and quality control (QC) samples in human RBC. Blank human RBC was screened prior to spiking to ensure it was free of endogenous interference at the retention time of VPA.

An eight-point standard curve ranging from 1 to 100 μg/mL of VPA having calibration standards at 1, 2, 5, 10, 20, 40, 80 and 100 μg/mL was prepared by spiking the blank RBC with appropriate amounts of VPA. QC sample were prepared at four concentration levels: 1 μg/mL, low limit quality control sample (LLOQ); 2 μg/mL, low quality control sample (LQC); 40 μg/mL, medium quality control sample (MQC), and 80 μg/mL, high quality control sample (HQC) for VPA, in a manner similar to the peak area ratios of the target ions of the drugs to those of the IS were compared with weighted (1/*y*) least-squares calibration curves in which the peak area ratios of the calibration standards were plotted versus their concentrations.

Lower limit of quantitation (LLOQ) was determined based on the criteria that the analyte response is at least ten times of base-line noise, and can be determined with precision of 20% and accuracy of 80-120%. The method was validated for selectivity, linearity, precision, accuracy and recovery.

Selectivity was performed by analyzing the blank RBC in human samples from different sources to test for interference at the retention times of analytes and internal standards. The intra-day and inter-day accuracy were determined by replicate analysis of QC sample and at LOQ were extracted from the sample batch. Accuracy is defined as the percent relative error (%RE) and assay precision was calculated by percent relative standard deviation (%RSD). The recovery of VPA in RBC was determined by comparing the response of the analytes extracted from replicate QC samples ($n = 4$) at LLQC, LQC, MQC, HQC with the response of analytes from post-extracted RBC standard samples at the equivalent concentration.

Clinical Pharmacokinetic Study. A total of ten healthy male subjects, weight 69.8 ± 10.5 kg, height 173.7 ± 5.9 cm and age 21.8 ± 3.0 years, were participated for the pharmacokinetic study at Clinical Trial Center of Chonbuk National University Hospital, Seoul, Korea. This protocol was approved by the Institutional Review Board of The Chonbuk National University Hospital and the samples were collected following subject's informed consent. All subjects received 500 mg of VPA orally twice a day on day 1-4 to establish steady-state pharmacokinetics. Blood samples were collected at pre-dose and at 60, 120, 180, 240, 300, 360, 480 and 720 min post-dose on day 4. The plasma was separated by centrifugation and kept at −80 °C until analysis. The partition coefficient of a given drug in red blood cells, $K_{RBC/PL}$, is determined by the ratio of the concentration of the compound in RBC (C_{RBC}) over that in the equilibrating plasma (C_{PL}).[10]

Results and Discussion

Chromatographic Separation and Mass Spectrometry. Most reported methods for the quantification of VPA concentrations in human RBC are based on GC/MS. In this laboratory we find it easier and more convenient to develop and validate a new LC-MS/MS method using protein precipitation rather than using GC/MS. The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution for the VPA and IS. The analytes were monitored by MRM of the deprotonated molecules at *m/z* 143 and 121 for VPA and IS, respectively. The MS parameters were optimized to obtain the highest signals for the $[M-H]^-$ ions (Table 1) and mobile phase 0.1% acetic acid (pH 4.0) (solvent A)/methanol (solvent B) was considered the best combination due to its high S/N ratio, considerable average peak width. Comparisons of the extraction efficiency by protein precipitation using methanol, acetonitrile and liquid-liquid extraction using *tert*-butyl methyl ether, dichloromethane and ethyl acetate from human RBC samples are not shown. For protein precipitation using acetonitrle, accuracy and precision is very low than methanol. On the other hands, for liquid-liquid extraction, three solvents (*tert*-butyl methyl ether, ethyl acetate and dichloromethane) need long and complex pretreatment time despite highly accuracy and precision

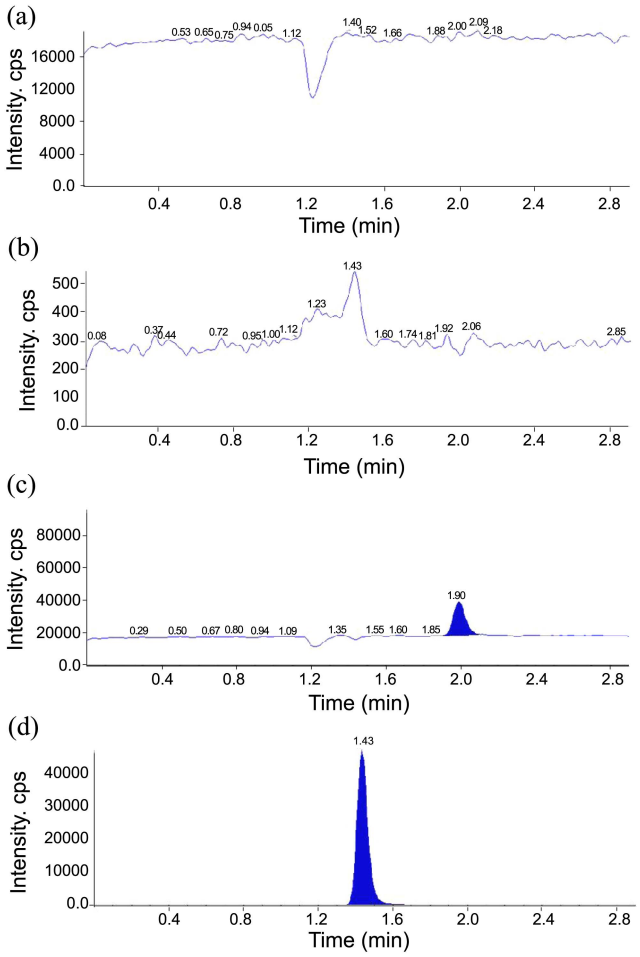

Figure 2. Representative chromatograms obtained from an extracted blank RBC sample and detection of analytes. (a) blank plasma with VPA, (b) blank plasma with benzoic acid, (c) LLOQ (1 μg/mL) of VPA (d) 100 μg/mL of benzoic acid.

Table 2. Intra-day and inter-day precision and accuracy (n=5) of VPA calibration standards in human RBC

Statistical variable	Concentration (μg/mL)								*r*
	1	2	5	10	20	40	80	100	
Intra-day									
Mean	0.97	1.93	4.89	10.68	20.78	39.72	80.36	98.70	0.9997
CV (%)	2.69	3.20	1.59	1.49	1.19	1.44	0.58	0.77	
Accuracy (%)	96.62	96.48	97.76	106.8	104.0	99.28	100.3	98.70	
RE (%)	0	-5.0	-2.0	7.0	4.0	-0.8	0.5	-1.3	
Inter-day									
Mean	0.98	1.91	4.97	10.46	20.32	40.20	80.70	98.44	0.9997
CV (%)	4.39	3.32	3.57	1.66	2.73	1.36	0.43	0.72	
Accuracy (%)	98.84	95.84	99.58	104.6	101.5	100.5	100.8	98.44	
RE (%)	0	-5.0	0	5.0	1.5	0.5	0.9	-1.6	

similar to methanol precipitation. In conclusion, RBC extraction of VPA and IS by protein precipitation (methanol) yielded the highest efficiency.

Method Validation. The calibration samples were prepared by spiking 50 μL control RBC with appropriate amounts of VPA and internal standard on the day of analysis. The lower limit of quantitation (LOQ) was defined as the lowest concentration at which the relative standard deviation from the nominal concentration was less than 20%. The analysis of blank plasma samples from six different sources did not show any interference at the retention times of VPA (2.0 min) and benzoic acid (1.4 min), confirming the specificity of the present method (Figure 2). The intra-day and inter-day precisions and accuracy of the method are summarized in Table 2-5.

Samples for the determination of precision were prepared by appropriately spiking control RBC in bulk to get concentrations of 1, 2, 40 and 80 μg/mL. Five replicates at each concentration were processed as described, in the sample preparation on day 0, 1, 2, 3, 4 and 5 to determine the inter-day and intra-day reproducibility. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision. The accuracy ranged from 96.62 to 106.80% for intra-day assay and 95.84% to 104.60% inter-day assay, respectively. Both intra-day and inter-day CV values ranged from 0.72 to 4.39% at four QC levels. The intra-day and inter-day RE values for VPA were −5.0 to 7.0% at four QC levels. The recovery of VPA from RBC ranged from 95.0 to 107.0% across the experimented eight concentrations. This method was successfully employed to analyze VPA in RBC and plasma pharmacokinetic samples. The accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the spiked/nominal concentration. We yielded 0.042 ± 0.007 as $K_{RBC/PL}$, partitioning ratio of human RBC and plasma (Figure 3). This study provided further evidence that the new method is comparable to the traditional method for RBC partitioning ratio measurement.

Table 3. Intra-day and inter-day recovery (n=5) of VPA in human RBC

Concentration (μg/mL)	Recovery (%)	
	Intra-day	Inter-day
1	100.0 ± 0.0	100.0 ± 0.0
2	95.0 ± 0.1	95.0 ± 0.1
5	98.0 ± 0.1	100.0 ± 0.2
10	107.0 ± 0.2	105.0 ± 0.2
20	104.0 ± 0.2	101.5 ± 0.6
40	99.3 ± 0.6	100.5 ± 0.5
80	100.5 ± 0.5	100.9 ± 0.4
100	98.7 ± 0.8	98.4 ± 0.7

Values are presented as Mean ± S.D

Table 4. Intra-day and inter-day precision and accuracy(n=5) of VPA in human RBC QC samples

Statistical variable	Concentration (μg/mL)			
	1	2	40	80
Intra-day				
Mean	1.02	2.05	42.18	85.76
CV (%)	3.29	3.35	0.86	0.73
Accuracy (%)	102.02	102.68	105.40	107.00
RE (%)	2.0	2.7	5.5	7.2
Inter-day				
Mean	1.01	1.97	39.78	81.02
CV (%)	2.46	4.02	1.73	2.88
Accuracy (%)	101.06	98.95	99.32	101.35
RE (%)	1.0	-1.5	-0.6	1.3

Table 5. Recovery (n=5) of VPA in human RBC QC samples at intra-day and inter-day

Concentration (μg/mL)	Recovery (%)	
	Intra-day	Inter-day
1	102.0 ± 0.0	101.0 ± 0.0
2	102.7 ± 0.1	98.5 ± 0.1
40	105.5 ± 0.4	99.5 ± 0.7
80	107.2 ± 0.6	101.3 ± 2.3

Values are presented as Mean ± S.D

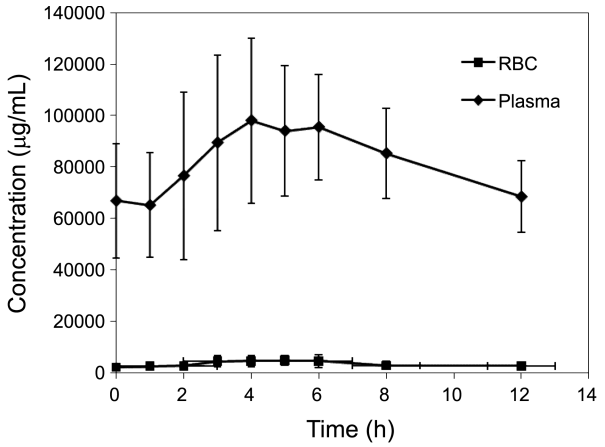

Figure 3. Mean plasma and RBC concentration-time profile of VPA after oral administration to ten male volunteers. Each point represents the mean ± S.D.

Conclusions

In summary, to our knowledge, this is the first method described for the validation of VPA in human RBC by LC-MS/MS in negative ESI mode using MRM, and validated. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (3 min) and rapid extraction. The LOD for VPA was 1 μg/mL using 100 μL human RBC. The use of reversed-phase column enabled good performance to be achieved in elution and separation. Thus, the present method may be useful for routine monitoring or pharmacokinetic studies on VPA in patients with epilepsy. A comprehensive therapeutic monitoring of anticonvulsant therapy with VPA demands the availability of suitable methods for measuring VPA concentrations.

A sensitive and rapid analytical method has been developed for measuring RBC partitioning coefficients for pharmaceutical compounds. The partition coefficient of a VPA in red blood cells, $K_{RBC/PL}$, is determined by the ratio of the concentration of the VPA in RBC (C_{RBC}) over that in the equilibrating plasma (C_{PL}). The method described here will be useful for screening compounds for potential high RBC partition, predicting potential drug toxicity, and investigating mechanisms associated with RBC partitions. In addition, the method will be applied to clinical research for study of distribution ratio of valproic acid in blood.

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