International Journal of Pharmacy Teaching & Practices 2013, Vol.4, Issue 2, 644-648.

Bioanalytical method validation of Valproic Acid in Human Plasma *In-vitro* after Derivatization With 2,4-Dibomoacetophenon by High Performance Liquid Chromatography-Photo Diode Array and its Application to *In-vivo* study

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Research Article

Please cite this paper as: Sri Teguh Rahayu[1], Yahdiana Harahap[2] dan Harmita[2]. Bioanalytical method validation of Valproic Acid in Human Plasma *In-vitro* after Derivatization With 2,4-Dibomoacetophenon by High Performance Liquid Chromatography-Photo Diode Array and its Application to *In-vivo* study. IJPTP, 2013, 4(2), 644-648.

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Abstract

Valproic acid is one of mostly used antiepileptic drug which have side effects, so it is highly recommended to evaluate its plasma concentration The aim of the research was to validate a method for the determination valproic acid in plasma in-vitro and in-vivo after derivatization with 2,4-dibromasetofenon using high performance liquid chromatography-photo diode array. Valproic acid and internal standard nonanoic acid were extracted from plasma sample with ethyl acetate, after that supernatan was neutralizatied and evaporated and dried residue reconsituted in derivate-catalyst solution then derivatized at 75ºC for 25 minutes. The resulting derivatives were separated on a Sunfire C_{18} (250 mm x 4.6, 5 µm) reverse phase column with acetonitrile-water (73:27) as mobile phase, were detected at 294 nm and analysis were run at flow rate 1.5 mL/minute. The calibration curve in plasma *in-vitro* (Y =0.0123 + 0.0085 x) presented good linier (r = 0.9999) between 4.75-237.75 µg/mL with LLOQ 4.75 µg/mL. The mean of relative recovery at low concentration, medium concentration and high concentration are 100.67%, 99.78%, and 93.16 %, respectively. Intra- and inter- day coefficient of variation and percent error value of the assay method not more than 15%. The presented method was might be applied to the determine of the valproic acid concentration in plasma after oral administration of 500 mg sodium divalproate.

Keywords: Valproic acid, derivatization, 2,4-dibromoacetophenone, High Performance Liquid Chromatography- Photo Diode Array, divalproic sodium, *in-vivo*

Introduction

Valproic acid (antiepileptic drug) is a chemical compound that can selectively depress the central nervous system and is used to prevent and treat evoked epilepsy (epileptic seizure) and non-epileptic attack without causing respiratory depression (Siswandono dan Purwanto, 2001).

Valproic acid is widely used as an anticonvulsant since 1978 in the singular or in combination with other drugs, such as with carbamazepin, phenitoin ar phenobarbital. US FDA in 1995 recommended valproic acid is used in the treatment of mania associated with bipolar disorder. Anticonvulsant effect of valproic acid were related to increased levels of GABA in brain, although the actual mechanism is not known. (Schwartz, Massa, Gupta, Al-Samrrai, Devit and Masand, 2000; Manoguerra, et al., 2008).

Agency for Food and Drug Administration Indonesian set same criteria for oral drugs past released dose form systemically off quickly in the list of mandatory bioequivalence trials. One of Criteria set when the drugs is intended to serious condition that requires a definite therapeutic response (critical use drugs), for example antituberculosa, antiretroviral, antimalaria, antibacteria, antihypertensi, antiangina, antiasma, heart failure drugs, and antiepileptic. Valproic acid is one of the antiepileptic drugs requiring equivqlence trial (Badan POM, 2004).

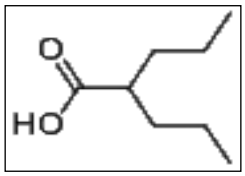
Valproic acid is very rapidly absorbed after oral administration, althrough valproic acid is a drug with a simple structure but its metabolit metabolites may cause thrombositophenia, neurotoxycity, dan hepatoxycity (Alsarra, Al-Qmar, and Belal, 2005).

Levels of valproic acid therapy is estimated to 50-150 µg/mL and the toxic levels estimated > 150 µg/mL. (Rompotis, Parissi-Poulou, Gikas, Kazanis, Vavayannis and Panderi, 2002; Bauer, 2008). Therapeutic levels can be achieved after a few weeks of delivery, while levels in the plasma after administration of a single dose of 500 mg tablet of sodium divalproat is ± 20 µg/mL. Given the use of valproic acid in epilepsy patients requiring long treatment time and side effect it is necessarry to develop a valid methodof analysis that can be used to determine levels of valproic acid in the blood so it

can be used to optimize therapy and testing for bioavailability and bioequivalence.

In this study, valproic acid analysis performed using the method of high performance liquid chromathograph-Photo Diode Array. This method is common method that is easy to follow and implement. Indonesian Pharmacope of the literatue mentions many chromatographic method is the main method used for analysis of drugs because it can be used for both qualitative and quantitave analysis (Chamberlain, 1985; Rohman, 2009).

Nonanoic acid used as internal standard, both valproic acid and nananoic acid they do not have uptake the UV-Vis absorption area. To improve detection in this study used 2,4-dibromoacethophenon the derivat, a compound which generally used the derivat for derivatization of carboxylic acids that are easy to obtain and 18-Crown eter-6 as a catalyts that help accelarate the derivatization reaction



Picture 1. Valproic acid

Material and Methodology

1. EXPERIMENTAL

1.1 Reagents

Divalproat sodium tablets (Abbott Indonesia), valproic acid purely as reference (Danisco), Nonanoic acidas internal standard (Sigma Aldrich), 2,4-dibromoacethophenon 98% as derivate (Sigma Aldrich), 18-Crown ether-6 99% as catalyts (Sigma Aldrich), acetonitril HPLC grade, potasium hydroxida, hexane, hydrochloride acid pro-analys grade (Merck®), aquabidestilata (Ika Pharmindo Farma®) dan human plasma (PMI).

1.2 Chromatographic Conditions

Valproic acid concentration in plasma were determined using high performance liquid chromatography (HPLC) Waters® Alliance 2695 separation module equipped detector (*photo diode array*) PDA Waters 2996 with autosampler, derivatization result injected into the system and separation were using Sunfire® C_{18} column (250 mm x 4.6, 5 µm) with isocration elution using a mobile fase acetonitril-water (73:27, v/v). The flow rate was 1.5 mL/min and the PDA detector wavelength was set at 294 nm.

1.3 Standard Solution

Stock solution of valproic acid (20 mg/mL) was prepared by dissolving 200 mg of valproic acid in 10 mL of methanol. Working solution of valproic acid were diluted form stock solution with methanol to 4.75 µg/mL, 11.89 µg/mL, 29.72 µg/mL, 59.44 µg/mL, 118.87 µg/mL dan 237.75 µg/mL for the calibration curve.

Stock solution internal standard nonanoic acid prepared by carefully weighing 90 mg nonanoic acid dissolved in 10 mL mehanol, to obtain a concentration of 9 mg/mL

Stock solution derivate of 2.4-dibromoacethophenon (20.000 µg/mL), prepared by carefully weighing the 2.4-dibromoacethophenon 200 mg dissolved in 10 mL acetonitril.

Catalyt solution of 18-crown ether 6 (1000 µg/mL), prepared by carefully weighing the 18-crown ether 6 (1000 µg/mL) 10 mg dissolved in 10 mL acetonitril.

2. PROCEDURE

3.1 Sample Preparation

Plasma was prepared from the blood of volunteer and stored at -20°C before analysis. 250 µL of plasma was transferred to centrifuge tubes, and 50 µL internal standard solution in concentration 1800µg/mL and 250 µL 0,5 M hydrochloride acid were added and vortex mixed for 5 second. 3 mL Ethyl acetate was added and extracted using a vortex for 3 minute, then centifuged 10 minute at 3000 rpm. After centrifugation, 2 mL of supernatant was transferred to clean glass tube and added potassium hydroxyde in methanol. The solvent was evaporated with nitrogen gas. The dry residue was derivatized according to the procedure and after cold as much as 30 µL injected into a high performance liqid chromatography.

3.2 Derivatization Procedure

Dried residue obtained was then reconsituted using derivate-catalyst solution 1.5 mL then derivatized at 75ºC for 25 minutes.

3. VALIDATION

3.1 Linierity

The linearity of an analytical method is its ability to elict test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range (USP 24).

Six calibration concentration levels were used (4.75 µg/mL, 11.89 µg/mL, 29.72 µg/mL, 59.44 µg/mL, 118.87 µg/mL and 237.75 µg/mL), in addition to the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS). The slope, intercept dan correlation coefficient (r) were determinated by method of regretion linier and the data are colleted.

3.2 LOQ

The limit of quantitation (LOQ) was determined as the lowest concentration of valproic acid in human plasma that could be quantified with acceptable precision and accuracy under the experimental conditions with a signal-to-noise ratio of at least 10:1.

3.3 Accuracy and Precision

Both the intra- and inter-day precision and accuracy of the method were determined by analysis of replicates (n = 5) of QC samples (14.57, 104.39, and 194.21 µg/mL). The precision of the method was described as relative standard deviation (RSD) among each assay. The accuracy of this analytic method was determined by the percent of mean deviation from known concentration, bias% = [(concentration found - known concentration) x 100/known concentration]. Precision and accuracy were calculated at each concentration.

3.4 Stability

The stability of valproic acid in plasma
was evaluated in five studies: a short term study, a long-term study, a freeze thaw study for 3 cycles, post-preparative stability (stable derivatives for 24 hours in autosampler) and stock solution stability. The QC samples low (14.57 µg/mL) and high (194.21 µg/mL) for valproic acid were assayed in triplicate. The concentration of valproic acid after each storage period was related to the initial concentration as determined for samples that were freshly prepared and processed immediately. The stability of standard stock solution was also tested after under refrigeration(4ºC).

3.5 Recovery

The extraction recovery of valproic acid in human plasma was evaluated by comparing the analyte to IS peak area ratios of processed QC samples (14.57, 104.39, and 194.21 µg/mL) with those from valproic acid standard solutions, which were similarly prepared and had the same final concentration except that methanol replaced blank plasma.

4.6. selectivity

Selectivity test is done at LLOQ concentration (14.57 µg/mL) using 6 diffferent plasma sources, each source plasma obtained was 2 replication and coefficient of variation (% CV)

Application

A healthy female volunteer was given 500 mg divalproat sodium oral tablet extended release. Blood sample (9 mL each) were obtained at 0, ½, 1, 1½, 2, 2½, 3, 3½, 4, 4½, 5, 6, 8, 12, 24, 48 and 72 hours after oral administration.
The blood was then slightly shaken and sentrifuged at 3000 rpm for 10 minute and the plasma sample were stored at -20°C before analysis. During the study,subjectcondition monitored by medical doctor.

Results and Discussion

FDA, 2001 the industry guidelines for bioanalytical method validation mentioned parameters for bioanalytical method validation include selectivity, accuracy, precision, recovery, and stability of the calibration curve of analyte in the sample. Both valproic acid and acid nonanoat including both carboxylic acid groups that do not have strong absorption in the UV-Vis absorption area, so the carboxylic acid groups must be converted into esters by reaction with aromatic halides, such as bromide fenasil (PBR), naftasil bromide (NBR) or other analog (Chamberlain, 1985; Blau and Halket, 1993). Therefore, derivatization can be part of the sample preparation, derivatization was performed with valproic acid prakolom procedure done after liquid-liquid extraction (Chamberlain, 1985). To improve the selectivity of the alkylation reaction sensititas and a catalyst such as carboxylic acid crown ether or tertiary amine. Crown ether complex cations, will increase the solubility of carboxylate anions in the solvent (Blau and Halket, 1993). At this derivatization process used catalyst 18-crown-6 ether, which is a compound consisting of 12 carbon atoms with 8 ties formed as a crown ether (crown) or crown ethers. 18-crown-6 ether works as a phase transfer catalyst which will bind cations of complex cation-carboxylate-cation forms a complex catalysts, aiming to increase the reactivity of carboxylate anion, so that the anion will be more free to react when derivatization process (Pine, Hendrikckson, Cram, Hammond, 1988).
Internal standard nonanoat acid and valproic acid can be well separated with chromatography systems used condition with retention time (retention time) on the 16th minute and 11 minutes. No endogenous compounds were found at the time of separation nonanoat acid with valproic acid. Total time required for the separation is 24 minutes. Good separation between acid nonanoat with valproic acid after derivatized shown in figure 1.
Mobile phase composition is an important factor in the separation, solvent mixture of acetonitrile-water (73:27) produced the best separation of valproic acid and acid that has been derivatized nonanoat because it produces a resolution of> 1.5 with a theoretical plate number (N)> 2500 and HETP as well as the small peak (peak) are symmetric.

TESTING METHOD VALIDATION

Comparison between the peak nonanoat acid and valproic acid in plasma after derivatization linear at 4,75 µg/mL– 237,75 µg/mL. Produce a calibration curve linear regression equation y = 0,0066 + 0,0090x with r = 0,9999 where x is the concentration of valproic acid and y is the area ratio valproic acid with nonanoat acid as internal standard.
Extraction method used in this study is the liquid-liquid extraction with ethyl acetat. Valproic acid is a potent drug compounds bound plasma proteins so that the extraction method is critical to the amount of valproic acid during the proccesn of extraction interested. In this way the values obtained recovery at low concentration of between 90.19% - 10.,74%,

medium concentration between 92.75% - 106.51% and high concentration between 90.87% - 99.38%.

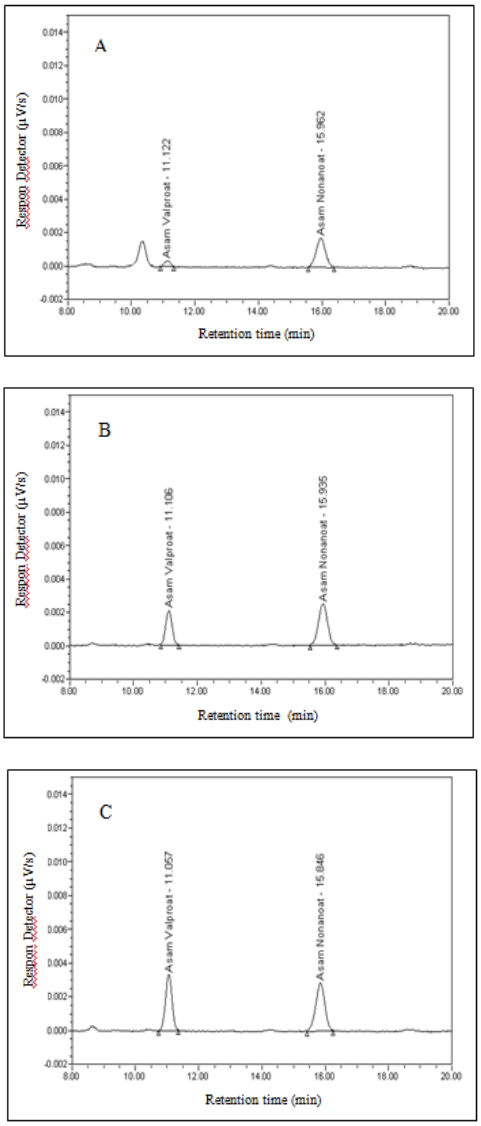


Figure 2. Chromatogram of valproic acid and nonanoic acid as internal standard at low concentration (A), medium concentration (B) and high concentration (C) elution used Sunfire® C_{18} column (250 mm x 4.6, 5 µm) with isocration elution using a mobile fase acetonitril-water (73 :27) fow rate 1.5 mL/min.

Precision and accuracy of valproic acid in QC samples (Table 1) fell within the limit of acceptability. All values were less than 9.5%. This validation demonstrates the reliability of our method.

Tabel 1. Precision and accuracy of the LC method for determining valproic acid concentrations in plasma samples.

Added concentration (µg/mL)	Intra-day (n = 5) Found (µg/mL) ±SD	RSD (%)	Bias (%)
14.57	14.90±0.79	5.31	2.26
104.39	102.36±4.96	4.85	-1.94
194.21	180.31±0.77	0.43	-7.15
14.57	14.79 ± 0.49	3.32	1.51
104.39	103.76 ± 3.07	2.96	-0.60
194.21	186.98± 6.64	3.55	-3.72

Stability results for valproic acid in plasma are shown in Table 2, indicating that valproic acid was stable in plasma samples under different storage conditions: immediately, after 24h at ambient temperature, after sample processing and being on the autosampler at 4ºC for 24 h, after three freeze-thaw cycles and after 69 days stored at -20 ºC. The stock solutions of valproic acid and IS were also stable after storage at refrigerator for 34 days. This suggests that valproic acid and IS are stable in experimental conditions.

Table 2. Stability of valproic acid in human plasma samples (n = 3).

Stability study	Added concentration (µg/mL)	Found (µg/mL)	RSD (%)
Short-term	14.57 194.21	14.49 181.94	1.07 0.71
Three free-thaw cycles	14.57 194.21	15.45 187.97	1.43 3.14
Autosampler stability for 24 h	14.57 194.21	14.44 181.69	3.05 0.17
Stability at -20 ºC for 69 days	14.57 194.21	15.32 205.6	2,72 3.90

APPLICATION FOR *IN-VIVO* STUDIES

In-vivo test results can be seen in Table 3 and 4 was known concentrations of valproic acid in plasma illustrates many valproic acid and absorbed into the systemic circulation. This study uses only one subject, which is itself intended as a preliminary research to determine whether the resulting methods can be applied in-vivo.

Elimination rate constant (Ke) is the slope / slope which can be determined by calculating the linear regression, linear regression equation Y = 3.1705-.0169 x, so that the known elimination rate constant (Ke) divalproat sodium tablets is 0.016 mg / / hour and a half-tablet of sodium divalproat (T ½) was 41.006 hours over 24 hours so that the administration as a single dose is appropriate. Absorption rate (Ka) is the slope (slope) linear regression equation concentration of 0.5 to 2 hours that have been extrapolated, the regression

equation Y = 3.290 to 0.579 x, so that the known absorption rate constant (Ka) tablet divalproat is 0,579 mg sodium / hour. It spent 6.369 hours to reach maximum concentration (Cmax) of 20.82 mg / mL, but in this study the approximate hour-tmax ± 6-8 concentrations in-vivo results only 15.21 mg / mL and AUC0-inff obtained 1381.868 mg / mL / hour. In addition to physiological factors such as gender, age and metabolic systems there are other factors that affect the absorption of sodium tablet divalproat the absorption of valproic acid sodium tablet dosage divalproat. Over a period of time there is the possibility of absorption of the long part of the drug is not released or released in other parts of the intestinal absorption is not great. Two possibilities that make its bioavailability decreases (Fagiolino, Martin, Gonzales and Malanga, 2007).

Table 3. *In-vivo* Assay using a healthy volunteer after received a single dose 500 mg of divalproat sodium oral tablet.

Time (hour)	Cp (µg/mL)	Ln Cp	d-AUC	AUC_{0-t}
0	0	0,000	0,000	0,000
0,5	5,16	1,641	1,290	1,290
1	6,29	1,839	2,863	4,153
1,5	17,96	2,888	6,063	10,215
2	14,94	2,704	8,225	18,440
2,5	17,63	2,870	8,143	26,583
3	17,14	2,841	8,693	35,275
3,5	15,22	2,723	8,090	43,365
4*	20,4	3,016	8,905	52,270
4,5	20,26	3,009	10,165	62,435
5	19,95	2,993	10,053	72,488
6	15,21	2,722	17,580	90,068
8	17,65	2,871	32,860	122,928
12	18,7	2,929	72,700	195,628
24	16,79	2,821	212,940	408,568
48	10,36	2,338	325,800	734,368
72	0	0,000	124,320	858,688

Table 4. Pharmacokinetic Parameters

Parameter	Sodium divalproic
Ke	0.0169
Ka	0.579
T1/2	41.006 hours
Tmax	6.369 hours
Cmax	20.82 µg/mL
$AUC_{0-\infty}$	1381.868 µg ,hour/mL

Conclusion

Analysis of in-vitro method valid in the range of the calibration curve 4,75 µg/mL– 237,75 µg/mL with LLOQ 4,75 µg/mL and can be applied for both in-vivo bioavalability or bioequivalence study becauce is meets the parameter of accuracy, precision, recovery, calibration curve, selectivity and stability established by the FDA Guidance for industry. Bioanalytical Method Validation, 2001

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AUTHORS' CONTRIBUTIONS

Authors contributed equally to all aspects of the study.

PEER REVIEW

Not commissioned; externally peer reviewed.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.