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Quantification of Anandamide and 2-Arachidonylglycerol in Plasma Samples: A Short, Non-toxic HPLC Method and Sample Storage

[Plazma Örneklerinde Anandamid ve 2-Araşidonilgliserol'ün Kantitatif Değerlendirmesi: Kısa, Nontoksik Bir HPLC Metodu ve Örnek Saklama]

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ABSTRACT

Objective: The endocannabinoid system plays an important modulatory role in brain physiology, pain sensation, appetite regulation, cardiovascular system, female reproductive system and the immune system. The endogenous ligands of cannabinoid receptors, anandamide (ANA) and 2-Arachidonylglycerol (2-AG) have been identified in various mammalian tissues. However, it difficult to quantify them accurately in blood and in tissues since these endogenous cannabinoids are found in small amount and they are metabolized very quickly. In order to quantify ANA and 2-AG from blood accurately, it was aimed (a) to determine pre-analytical conditions in blood sampling procedure, (b) to improve extraction process and quantification of ANA and 2-AG in plasma samples by HPLC, and (c) to determine storage conditions and period of these metabolites.

Method: Rat blood samples were obtained by intra-cardiac puncture and centrifuged immediately. ANA and 2-AG plasma levels were determined with the PMB-Immobilized Beads Affinity Chromatography Method followed by HPLC analysis.

Results: Immediate centrifugation at 0°C prevented artificial synthesis of ANA and 2-AG. Derivatization step using toxic chemicals was omitted. Furthermore, the volume of the plasma samples was decreased from 3 ml to 1 ml plasma. This may allow designing longitudinal rat studies without the termination of the animals. For the first time, it was shown that ANA and 2-AG could be stored as plasma and ethanol extracts for different time periods at -80°C at least for one week.

Conclusion: The measurement protocol was simplified and safer. In addition, sample volume was decreased and storage conditions were optimized.

Key Words: Anandamide, 2-arachidonylglycerol, plasma, sample storage, nontoxic HPLC method

ÖZET

Amaç: Endokannabinoid sisteminin beyin fizyolojisi, acı algısı, iştahın düzenlenmesi, kardiyovasküler sistem, kadın üreme sistemi ve bağışıklık sistemi üzerinde düzenleyici etkisi bulunmaktadır. Kannabinoid reseptörlerinin endojen ligandları anadamid (ANA) ve 2-araşidonilgliserol (2-AG) bir çok memeli dokusunda tespit edilmiştir. Ancak bu endojen kannabinoidler dokularda ve kanda az miktarda bulunmaları ve çok hızlı metabolize olmaları miktarlarının doğru ölçülmesini zorlaştırmaktadır. Bu çalışmanın amacı ANA ve 2-AG'nin kandan hatasız ve doğru ölçülebilmesi için (a) kan örneklerinin elde edilmesinde analitik ölçüm öncesi şartların belirlenmesi, (b) ANA ve 2-AG'nin plazma örneklerinden ekstraksiyonu ve HPLC yöntemi ile nicel olarak belirlenmesinin geliştirilmesi, ve (c) bu metabolitlerin saklama koşulları ile süresinin belirlenmesini içermektedir.

Yöntem: İntrakardiyak aspirasyon yöntemiyle elde edilen sıçan kan örnekleri bekletilmeden santrifüj edilmiştir. ANA ve 2-AG plazma düzeyleri "PMB-İmmobilize Küre-cikli Seçici Kromatografi Yöntemi (PMB-Immobilized Bead Affinity Chromatography Method)" ni takiben HPLC yöntemi ile ölçülmüştür.

Bulgu: Kan örneklerinin 0˚C derecede santrifüj edilmesinin yapay ANA ve 2-AG artışını önlediği tespit edilmiştir. Toksik kimyasalların kullanıldığı derivatizasyon basamağı yöntemden çıkarılarak ölçümlerde kullanılan plazma miktarı 3 ml'den 1 ml'ye indirilmiş, böylece hayvanların sonlandırılmadan uzun süreli çalışmalarda kullanılmasına olanak sağlanmıştır. Ayrıca plazma ve etanol içerisinde ki ANA ve 2-AG'nin -80˚C derecede saklama sürelerinin farklı olsa da en az 1 hafta boyunca bozulmadan korunabildiği ilk kez bu çalışmada gösterilmiştir.

Sonuç: Ölçüm metotu basitleştirilerek daha güvenli hale getirilmiştir. Buna ek olarak örnek hacmi azaltılmış ve saklama koşulları optimize edilmiştir.

Anahtar Kelimeler: Anandamid, 2-Araşidonilgliserol, plazma, madde kararlılığı, nontoksik HPLC metodu

Introduction

The endocannabinoid system is a complex and well orchestrated network that includes at least two cannabinoid receptors (CB1 and CB2) identified in the central [1] and peripheral nervous systems [2- 4], enzymes taking part in anabolic and catabolic metabolism such as monoacylglycerol lipase and fatty acid amide hydrolase, and finally transporters carrying these molecules to tissues of interest. This system is regulated by endogenously synthesized lipid derivatives called endocannabinoids. The first endocannabinoids, anandamide (ANA) and 2-arachidonylglycerol (2-AG) have been identified in various mammalian tissues [5- 8]. Several others have been isolated so far; however, there is still a need for detailed studies to identify their function in living organisms. It has been clearly shown that ANA and 2-AG play important modulatory roles in brain physiology [9], pain sensation [1, 10], appetite regulation and obesity [11], immune response [12, 13], cardiometabolic risk [14] and female reproductive health [15, 16]. Since variations in the levels of endocannabinoids are determinants of disorders, the potential therapeutic impact of endocannabinoid related drugs have become a popular topic. For these reasons, clinical application of lipidomics to help determine and quantify these circulating endocannabinoids plays an important role in the prevention and treatment of these disorders and was studied by many scientists.

The biosynthetic pathways of the endocannabinoid ANA was also reviewed in the literature [17]. Since ANA and 2-AG have important roles in the regulation of various physiological processes, it is important to develop accurate extraction and quantification methods for the endocannabinoids ANA and 2-AG. Several methods are currently used to quantify endocannabinoids including (a) HPLC [18, 19], (b) HPLC/ RAD [20], (c) HPLC/MS [21] and (d) HPLC/MS-MS [22]. However, these sophisticated methods are rarely accessible and extremely expensive. The rationale in improving the extraction and quantification methodology of conventional HPLC will enable us to measure ANA and 2-AG precisely.

Extraction and quantification of endocannabinoids requires partial purification and chemical derivatization of the endocannabinoids [1]. Since ANA and 2-AG are found at very low concentrations (pmol/ml plasma) in biological samples, it is difficult to use the purification step including organic solvent extraction followed by thin-layer chromatography (TLC) or silica gel column chromatography. Pre-purification steps of ANA and 2-AG are variable and time-consuming.

A recent study developed a Polymyxin B-immobilized bead affinity chromatography technique combined with High-performance liquid chromatography (HPLC) [23]. Using affinity chromatography instead of organic solvent extraction and HPLC instead of GC/MS simplified ANA and 2-AG measurements. However, it contains the use of toxic chemicals in the derivatization and quantification steps.

The aim of this study was (a) to detemine pre-analytical conditions without exvivo synthesis in reduced volume of plasma, (b) to improve the Polymyxin B-selective adsorption followed by HPLC methodology to quantify ANA and 2-AG concentration in plasma by omitting the derivatization step and (c) to identify storage time and conditions of the extracted metabolites.

Material and Methods

Sample

The studies were carried out in accordance with the Guiding Principles in the Use of Animals in Experiments as adopted by the Hacettepe University Ethical Committee with the certification on 05/09/2006 and approval number 2006/53-8. Blood samples were collected by intracardiac aspiration into tubes containing 4.5 nM EDTA.

PMB-Immobilized Beads Affinity Method

The plasma was obtained by centrifugation of blood samples at 2000 g for 10 minutes at 0°C. ANA and 2-AG were isolated from plasma samples by PMB-Immobilized Beads Affinity Method modified from Wang [23].

1 mM phenylmethylsulfonylfluoride (PMSF) was added to 1 ml plasma in order to inhibit fatty acid amide hydrolase (FAAH) activity. The plasma was diluted with 3 ml ice-cold saline solution (isotonic NaCl solution, 0,9% w/v). 1 ml of 50% Affi-Prep Polymyxin B suspension was added and shaken for 1 hour at 4°C. After the binding process, beads were transferred to a microchromatography column (0.5 × 3 cm) and washed twice with 3 ml ice-cold saline solution. Remaining saline was removed by centrifugation at 5000 g for 3 minutes at 0°C. Anandamide and 2-AG were eluted from the beads by applying two times 200 µl of ice-cold absolute ethanol and centrifugation at 5000 g for 3 minutes at 0°C. The extraction step was carried out on ice and the extracts were kept at -20°C up to the HPLC injections through the bench work, otherwise at -80°C.

HPLC Analysis

The ANA and 2-AG contents of plasma samples were quantified by HPLC according to the procedures modified from Wang (23) and Koga (24). The HPLC System (Varian ProStar, United States) equipped with a Prostar Pump 240, ProStar PDA Detector 330 and ProStar Autosampler 410 (VARIAN) was used. Separation was carried out on a TSK gel ODS 80 TM (75 mm × 4.6 mm ⌅ 5 µm) column (TOSOH, Tokyo, Japan) with an elution solution of acetonitrile and water (80:20, v/v) at a flow rate of 1 ml/minute. Absorbance was monitored at 204 nm wavelength. 100 µl of the eluted sample from the PMB-immobilized beads column was injected to the HPLC. The

ANA and 2-AG standards were purchased from SIGMA. The stock standard solutions were prepared with ice cold absolute ethanol and the all dilutions were done with ice cold ethanol. All samples were examined as duplicates and quantified with double injection. The quantification of the ANA and 2-AG contents of the samples were based on the standard curve ranging from 25 pmol to 8 nmol/ml.

Precision and Accuracy

The precision of the procedure was evaluated by analyzing the standard mix with respect to the recovery of the spiked standard, intra-run repeatability and between-day repeability. LOD and LOQ were found as 15 and 50, respectively (S/N is 5). The intra-run repeatability was determined by analyzing ANA and 2-AG in 4nmol concentrations in 6 consecutive injections in the same day. The coefficient of variation (CV%) for ANA and 2-AG concentrations were found as 3.25 % and 4.75 %, respectively. The between-day repeatability was determined by analyzing the same standard mix in 5 consecutive days and CV% values of ANA and 2-AG concentrations were calculated as 3.25 % and 4.75% , respectively.

The spiked plasma samples were analyzed according to the procedure described above. The accuracy was determined by comparing the results from analyses of spiked plasma samples with results from analyses of standard mix in the known concentration. The recoveries were found as 96.22 % and 96.97 % for ANA and 2-AG, respectively.

Results and Discussion

This study was achieved to develop a sensitive method for the extraction and quantitative determination of the endocannabinoids ANA and 2-AG from plasma without any *exvivo* artificial synthesis. First, pre analytical conditions were determined following a modified HPLC method by eliminating the time consuming and toxic derivatization procedure which contains hazardous chemicals. Second, we reduced the sample volume to 1 ml in order to give the opportunity for longitudinal rat or mice studies. Finally, we determined the suitable storage time and conditions for ANA and 2-AG stored as either plasma or ethanol extracts.

Extraction and quantification of ANA and 2-AG in peripheral circulation is challenging. Handling and storage conditions after blood sampling play important role in accurate quantitative profiling of the circulating endocannabinoids. Several studies tried to explain ex vivo synthesis of ANA and 2-AG from erythrocytes at different temperatures ranging from 4°C to room temperature [25, 26]. Therefore, collecting blood in to the suitable tube and immediate centrifugation at 0°C is a critical step and is strictly recommended in endocannabinoid quantification. In this study, ANA and 2-AG plasma concentrations did not fluctuate due to the standardized pre-analytical protocol.

In previous studies, the volume of the plasma used for endocannabinoid determination was at least 2 ml [21, 23]. To obtain 3ml plasma from the rat and mice causes termination. In this study 500 µl to 1 ml of plasma was used. This approach gave the opportunity to work samples in duplicates without the need of termination of the animals. We not only reduced the plasma volume, but also modified the percent of plasma of the sample solution. Reducing the plasma volume to 1 ml and diluting it with 3 volumes of ice-cold saline solution improved selective binding affinity of the PMB-immobilized beads to ANA and 2-AG. Furthermore, it prevented intense interference of other lipids and proteins in the plasma. A clear separation between elutes by HPLC was observed (Figure 1). Diluted plasma naturally prevented non-selective binding and improved the affinity of ANA and 2-AG to the beads resulting in cleaner samples. We noticed that binding efficiencies of ANA and 2-AG on PMB-immobilized beads were excellent as we could not measure any ANA or 2-AG concentrations in the washing saline solution. ANA and 2-AG were not measured in the third washing solution indicating that two times washing with 3 ml saline was sufficient to detect the present metabolites.

Following these optimizations, ANA and 2-AG appeared as two separate peaks, in the fractionation step, with the retention times 3.2 and 3.9 minutes, respectively (Figure 1). There was a small shift in the retension times of ANA and 2-AG between the plasma and the commercial standard which was negligible and confirmed with spiking the samples with the known concentrations of the commercial standard.

The endocannabinoids ANA and 2-AG obtained from

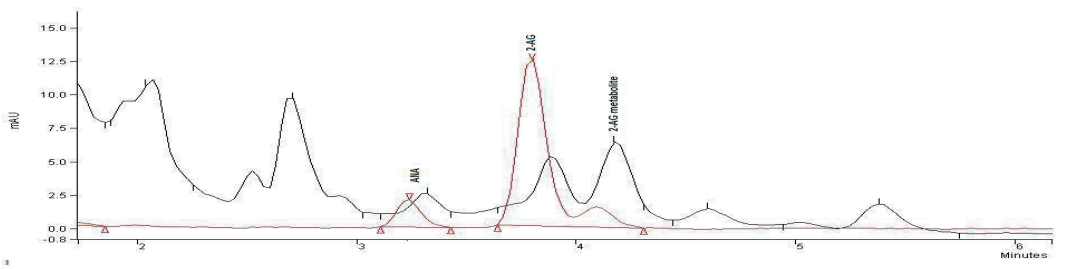

Figure 1. Chromatogram of rat plasma sample (Black line) and the synthetic standards for ANA and 2-AG (red line). ANA has a retention time of 3.2 minutes whereas 2-AG has a retention time of 3.9 minutes. The 2-AG metabolite appears as a second peak after 2-AG with a retention time of 4.2 minutes as shown on the sample chromatogram.

plasma were identical to the chromatogram of the standards where 2-AG appeared as two peaks labeled as 2-AG and its metabolite. Although 2-AG binds to CB1 and CB2, its metabolites do not show any affinity to these receptors and are known to be biologically inactive [27]. Therefore, it was aimed to determine 2-AG separately from its metabolite. Interestingly, concentration of 2-AG metabolite was higher in the plasma samples compared to the standards which may be explained with enzymatic degradation of 2-AG in literature [28]. PMSF was used to inhibit the FAAH enzyme which takes part in the breakdown of ANA and 2-AG. The concentration of 2-AG metabolites were always higher than that of the 2-AG itself indicating that PMSF was not able to prevent the breakdown of 2-AG. Previous studies showed that PMSF was able to inhibit the breakdown of 2-AG by only 80% and additional enzymatic activity, possibly monoacylglycerol lipase (MGL) was responsible for 2-AG degradation. MGL activity was inhibited by methyl arachidonoyl fluorophosphonate (MAFP) [29].

The derivatization step using 4-(N-chloroformylmethyl-N-methyl) amino-7-N,N-dimethylaminosulphonyl 1-2, 1,3-benzoxadiazole (DBD-COCl) dissolved in benzene at 60°C for 40 minutes as described by Wang [23] and Koga [24] was also mimicked in this study, however, this procedure resulted in bulky samples with a phase separation. Injection of these samples caused a rise in the column atmospheric pressure over the tolerable levels after around the 15th sample resulting in the shortened column life time. Moreover, the derivatization did not increase the sensitivity of the measurements. Since it was possible to detect ANA and 2-AG in biological levels in the fractionation step, we eliminated this derivatization process. By the quantification of ANA and 2-AG without derivatization, it was possible to avoid the usage of hazardous chemicals and shorten the time of the HPLC method for at least 1 hour.

Storage of blood samples can endanger accurate quantitative profiling of the circulating endocannabinoids. Although studies tried to determine the effect of freezing and thawing on plasma and blood samples in endocannabinoid quantification [26], there is a lack in the storage conditions and period for these molecules in literature. Therefore, we tried to find suitable storage conditions and time periods for ANA and 2-AG. Since blood cells synthesize endocannabinoids in ex vivo conditions [25] we tried to store the samples either as plasma or ethanol elutes. All plasma samples were pooled and standards of ANA and 2-AG were added. Samples were divided into seperate tubes so that each tube contained 1 ml of plasma. The first group of samples were measured on day 1 as triplicate with double injections. The rest of these tubes were divided into two equal halves. Half of these tubes dipped into liquid nitrogen and stored directly as plasma at -80°C, whereas the rest of the tubes were extracted to ethanol by the procedure described above and then dipped into liquid nitrogen before they were stored as ethanol elutes at the same conditions throughout the experiment until forthcoming measurement days. The ANA and 2-AG content of the samples were determined and measured periodically for two weeks and the results of 1, 8, 10 and 18 days were given in Table 1.

It was observed that ANA and 2-AG remained stable for different time courses depending on being stored in plasma or ethanol at -80°C (Figure 2). ANA was stable for at least 10 days when it was stored in plasma, on the other hand it started to decrease after 8 days while it was stored in ethanol (Figure 2 a, b).

Conclusion

Post handling steps and pre analytical conditions play important roles in the quantification of circulating endocannabinoids. Therefore, immediate centrifugation at 0°C is highly recommended in order to prevent artificial synthesis that masks the actual level of these metabolites. With simple optimizations, it was possible to decrease the sample volume up to 500 µl and thereby preventing non selective binding by improving the affinity. This study showed that exclusion of the derivatization step in the HPLC method eliminates the usage of hazardous and

Table 1. ANA and 2-AG stored at -80^{0}C as plasma and/or ethanol elute.

TIME OF MEASUREMENTS		Day 1 (Mean ± SD)	Day 8 (Mean ± SD)	Day12 (Mean ± SD)	Day 18 (Mean ± SD)
STORED AS PLASMA	ANANDAMIDE (nmol/ 1 ml plasma)	5.14 ± 0.24	5.27 ± 0.20	5.30 ± 0.08	4.19 ± 0.53
	2-ARACHIDONYLGLYCEROL (nmol/ 1 ml plasma)	5.01 ± 0.29	4.93 ± 060	4.92 ± 0.59	3.60 ± 1.24
STORED IN ETHANOL	ANANDAMIDE (nmol/ 1 ml plasma)	----------	5.81 ± 0.19	4.55 ± 0.22	3.88 ± 0.55
	2-ARACHIDONYLGLYCEROL (nmol/ 1 ml plasma)	----------	5.19 ± 0.44	4.98 ± 0.77	5.08 ± 0.17

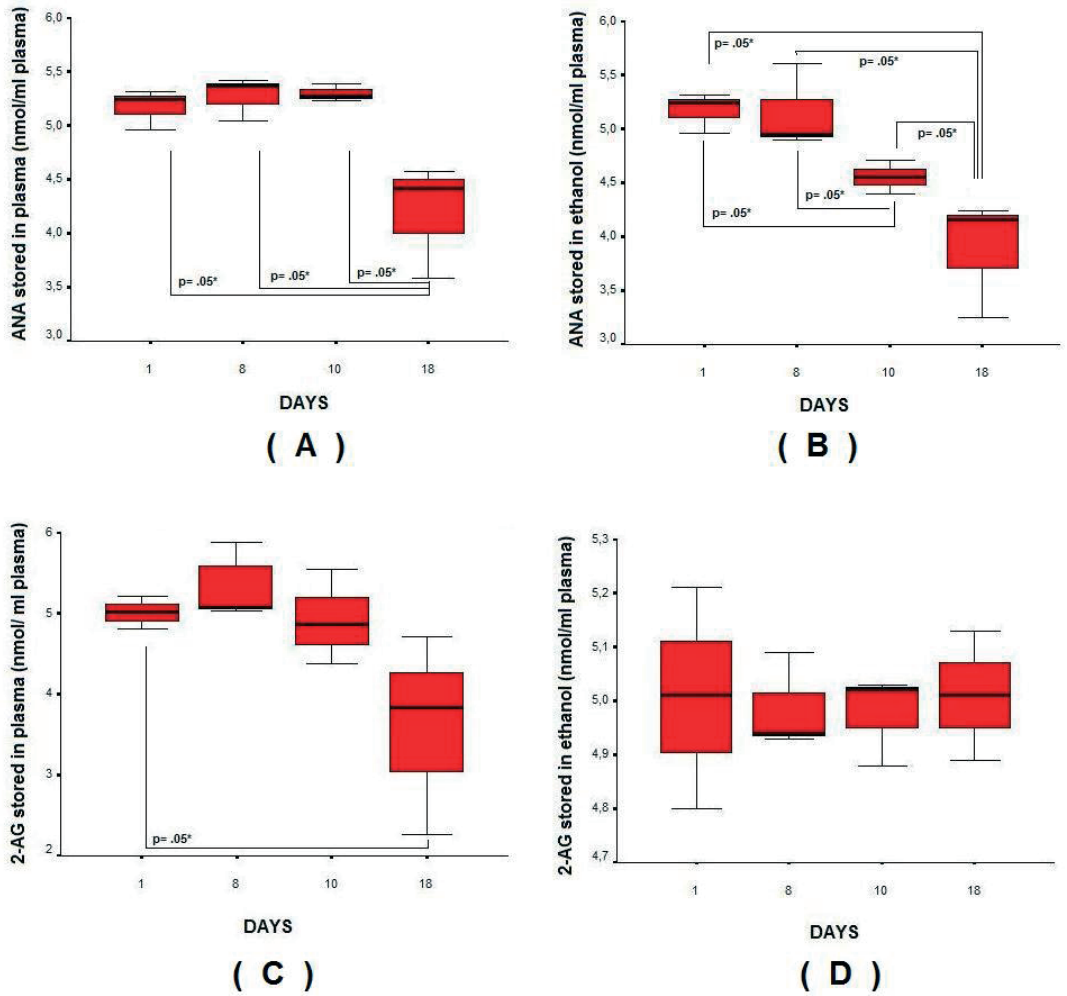

Figure 2. (a) ANA stored in plasma did not show any statistically significant difference between the measurements in 1, 8 and 10 day time points. On the other hand, ANA concentration measured at the 18th day was statistically decreased when compared to the measurements obtained on the 1st day (p = .05), 8th day (p = .05) and 10th day (p = .05). (b) ANA stored in ethanol was stable at least for 8 days and started to decline gradually on the 10th day. There was not any significant difference between the 1st day and the 8th day, but ANA was a significantly declined on the 10th day (p = .05) and 18th day (p = .05). (c) The only statistically significant difference in 2-AG concentartions stored in plasma was between the 1st day and the 18th day measurements (p = .05). (d) 2-AG stored in ethanol did not show any statistically significant difference at any measured time point.

toxic chemicals like benzene and DBD-COCl and shortens the experiment time at least for 1 hour. For the first time, we showed that ANA and 2-AG could be stored as plasma and ethanol extracts for different time periods at -80°C. The importance of the cold ring through the extraction steps and elution into the ice-cold ethanol were underlined. As a summary, the measurement protocol was simplified and become safer. In addition, sample volume was decreased and storage conditions were optimized.

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REFERENCES

[1]. Pertwee RG. Pharmacology of cannabinoid receptor ligands. Curr Med Chem. 1999;6(8):635-664.

[2]. Brown SM, Wager-Miller J, Mackie K. Cloning and molecular characterization of the rat CB2 cannabinoid receptor Biochim. Biophys. Acta. 2002;1576:255-264.

[3]. Grotenhermen F. Pharmacology of cannabinoids. Neur. Endocrinol. Lett., 2004;25:14-23

[4]. Klein TW, Newton C, Larsen K, Lu L, Perkinss I, Nong L, Friedman H. The cannabinoid system and immune modulation J Leukoc Biol**,** 2003;74:486-496

[5]. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA,

Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. Structure of a cannabinoid receptor and functional expression of a cloned cDNA. Science. 1992;258;1946-1949.

[6]. Matsuda LA, Lolait SJ, Brownstein M J, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of a cloned cDNA Nature. 1990;346:561-564.

[7]. Mechoulam R, Fride E, Di Marzo V. Endocannabinoids Eur J Pharmacol. 1998;359:1-18.

[8]. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids Nature. 1998;365:61-65.

[9]. Jiang W, Zhang Y, Xiao L, Van Cleemput J, Ji SP, Bai G, Zhang X. Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. J Clin Invest. 2005;115(11):3104-3116.

[10]. Richardson JD, Aanonsen L, Hargreaves KM. Antihyperalgesic effects of spinal cannabinoids Eur. J. Pharmacol. 1998;19:145-153.

[11]. Di Marzo V, Matias I. Endocannabinoid control of food intake and energy balance. Nat. Neurosci. 2005;8(5):585-589.

[12]. Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, Le Fur G, Casellas P. Cannabinoid-receptor expression in human leukocytes. Eur J Biochem. 1993;214(1):173–180.

[13]. Galieque S, Mary S, Marchand J, Dussossoy D, Carrière D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. Eur J Biochem. 1995;232(1):54–61.

[14]. Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, Di Marzo V. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. Int J Obes (Lond) 2007;31:692–9.

[15]. Janero DR, Makriyannis A. Targeted modulators of the endogenous cannabinoid system: future medications to treat addiction disorders and obesity. Curr Psychiatry Rep. 2007;9:365–73.

[16]. Maccarrone M. CB2 receptors in reproduction. Br J Pharmacol. 2008;153:189–98.

[17]. Okamoto Y, Wang J, Morishita J, Ueda N. Biosynthetic pathways of the endocannabinoid anandamide. Chem Biodivers. 2007;4(8):1842-1857.

[18]. Maccarone M, Barboni B, Paradisi A, Bernabo N, Gasperi V, Pistilli MG, Fezza F, Lucidi P, Mattioli M. Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. J Cell Sci. 2005;118(Pt 19):4393-4404.

[19]. Schmidt A, Brune K, Hinz B. Determination of the endocannabinoid anandamide in human plasma by high-performance liquid chromatography. Biomed Chromatogr. 2006;20(4):336-342.

[20]. Yang W, Ni J, Woodward DF, Tang-Liu DD, Ling KH. Enzymatic formation of prostamide F2alpha from anandamide involves a newly identified intermediate metabolite, prostamide H_2. J Lipid Res. 2005;46(12):2745-2751.

[21]. Giuffrida A, Rodriques de Fonseca F, Piomelli D. Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. Anal. Biochem. 2000;280(1):87-93.

[22]. Liu J, Harvey- White J, Osei-Hyiaman D, Razdan R, Gong Q, Chan A C, Zhou Z, Huang B X, Kim HY, Kunos G. A biosynthetic pathway for anandamide. Proc Natl Acad Sci U S A. 2006;103(36):13345-50.

[23]. Wang Y, Liu Y, Ito Y, Hashiguchi T, Kitajima I, Yamakuchi M, Shimizu H, Matsuo S, Maruyama I. Simultaneous measurement of anandamide and 2-Arachidonylglycerol by polymyxin B-selective adsorption and subsequent high-performance liquid chromatography analysis: Increase in endogenous cannabinoids in the sera of patients with endotoxic shock. Anal. Biochem. 2001;294:73-82.

[24]. Koga D, Santa T, Hagiwara K, Imai K, Takizawa H, Nagano T, Hirobe M, Ogawa M, Sato T, Inoue K, et al. High-performance liquid chromatography and fluorometric detection of arachidonylethanolamide (anandamide) and its analogues, derivatized with 4-(N-chloroformylmethyl-N-methyl)amino-7-N,N-dimethylaminosulp honyl-2,1 ,3- benzoxadiazole (DBD-COCl). Biomed. Chromatogr. 1995;9:56-67.

[25]. SVogeser M, Hauer D, Azad SC, Huber E, Storr M, Schelling G. Release of anandamide from blood cells. Clin. Chem. Lab. Med. 2006;44: 488-91.

[26]. Wood JT, Williams JS, Pandarinathan L, Courville A, Keplinger MR, Janero DR, Vouros P, Makriyannis A, Lammi-Keefe CJ. Comprehensive profiling of the human circulating endocannabinoid metabolome: clinical sampling and sample storage parameters. Clin Chem Lab Med. 2008;46(9):1289-95.

[27]. Sugiura T, Kondo S, Kishimoto S, et al. Evidence that 2-rachidonoylglycerol but not Npalmitoylethanolamine or anandamide is the physiological ligand for the cannabinoid CB2 receptor. Comparison of the agonistic activities of various cannabinoid receptor ligands in HL-60 cells. J Biol Chem. 2000; 275(1):605–12.

[28]. Savinainen JR, Saario SM, Niemi R, Jarvinen T, Laitinen JT. An optimized approach to study endocannabinoid signaling: evidence against constitutive activity of rat brain adenosine A1 and cannabinoid CB1 receptors. Br. J. Pharmacol. 2003;140:1451-1459.

[29]. Savinainen JR, Jarvinen T, Laine K, Laitinen JT. Despite substantial degradation, 2-arachidonoylglycerol is a potent full efficacy agonist mediating CB1 receptor-dependent G-protein activation in rat cerebellar membranes. Br. J. Pharmacol. 2001;134:664 – 672.