Targeted proteomics of cannabinoid receptor CB1 and the CB1b isoform

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Abstract

Cannabinoid receptors (CBR), including CB1 and CB2 have been therapeutic targets for a number of conditions. Recently, splice variants of the CB1R have been identified in humans. The isoforms differ in their N-terminus sequence and pharmacological activity relative to the CB1R, as a result, the differentiation between the CB1 receptor and its isoform is required. As a result, a selected reaction monitoring mass spectrometry (SRM-MS) method was developed for the quantitation of CB1 and the CB1b isoform in CHO cells transduced with CB1 and CB1b. The SRM-MS protocol was assessed with isotopically labeled peptide standards and had high reproducibility of intra-day assay (CVs from 1.9 to 4.3% for CB1 and 0.5 to 5.9% for CB1b) and inter-day assay (CVs from 1.2 to 5.2% for CB1 and 1.2 to 6.1% for CB1b).

Keywords

1. Introduction

The endocannabinoid system consists of the cannabinoid receptors (CBR), endocannabinoids and the enzymes responsible for synthesizing and degrading ECs. CBs are present primarily in brain and in lesser amounts in other parts of the body. The CB is a 45–53 kDa integral membrane G-protein-coupled receptors (GPCRs) containing seven transmembrane domains. To date, three subtypes of cannabinoid receptors have been identified which are CB1R [1], CB2R [2,3] and GPR55 [4]. These GPCRs play an important role in many physiological processes, including metabolic regulation, craving, pain, anxiety, bone growth, and immune function [5,6]. CB1R and CB2Rs mediate their effects via a pertussis toxin-sensitive GTP-binding regulatory protein Gi, while GPR55 does not couple to G(i/o) or G(q) but to Gα13 [7,8]. CB1R are abundant in the central nervous system particularly in regions such as those involved in cognition and short term memory and in motor function and movement [9,10]. They are also expressed in peripheral tissues including immune cells, heart, lung, bone marrow and gastrointestinal tissues [1], where they play major roles in glucose metabolism, cell proliferation, inflammation and apoptosis [3]. In islets of Langerhans the CB1 receptors are reported to be expressed in β cells, and/or α and

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δ cells [3,5,6,11,12]. As a result, CB1R is a therapeutic target for many conditions including inflammatory bowel disease, depression, metabolic syndrome and cerebral ischemia [3]. More recently, splice variants of the CB1R have also been identified in humans (CB1aR and CB1bR) [9], where they differ in their potency of action [13,14] These isoforms differ in their N-terminus sequence affecting their pharmacologic properties [9,10], therefore the identification and localization of the CB1 receptor and its isoform would be beneficial. Blocking the CB1 receptor in the periphery has been demonstrated to regulate insulin secretion and action [14–16]. While, CB1R is expressed in the central nervous system and peripheral tissue, the isoform CB1a is predominantly expressed in the central nervous system, although its levels are negligible relative to central CB1. CB1b, however, is the main isoform expressed in hepatocytes and is highly expressed in pancreatic β cells, where it regulates adenylyl cyclase activity [14]. Thus it is important for the endocannabinoid field to develop methods to distinguish CB1 and CB1b.

Currently, reverse transcriptase-polymerase chain reaction, western blot analysis, and Immune-histochemistry are the predominant method for the analysis of the cannabinoid receptors. However, the antibodies developed for the CB1 receptor have been fraught with non-specificity. For example, commercial antibodies used in the western blot analysis of CB1 transfected cells, brain lysates and rodent tissues result in multiple bands [17]. Further, we are unable to distinguish between CB1 receptor and its isoforms by western blot analysis. Thus, a targeted proteomic approach for the quantitation of CB1 and CB1b in a cellular matrix was developed and applied to CHO cells transduced with CB1 and CB1b isoforms.

2. Materials and method

2.1. Reagents

DMEM/F-12 medium was purchased from Invitrogen, 10% FBS from HyClone, GE Healthcare. Urea, Thiourea, Sodium Chloride, Sodium Carbonate, Silver Nitrate, Tris, Dithiothreitol, Ammonium Bicarbonate, TritonX-100, Protease Inhibitor cocktail, Trichloroacetic acid, Iodoacetamide were purchased from Sigma Aldrich. Sodium Thiosulphate, Potassium Ferricyanide, CHAPs were purchased from Fischer Scientific. LDS sample buffer and Puromycin was purchased from Invitrogen. Trypsin and Protease Max was purchased from Promega. Polybrene was purchased from Santa Cruz (Dallas). Lentivirus was purchased from Genecopia (Rockville, MD). 4–12% "NuPAGE® Novex® 4–12% Bis-Tris" 1 mm gel was purchased from Thermo Fischer Scientific. 2D quant kit was purchased from GE healthcare. Acetonitrile, Ethanol, Acetic Acid, Methanol were purchased from Sigma Aldrich

2.2. CHO cell transduction and culture

CHO cells were maintained in DMEM/F-12 medium (Invitrogen, Grand Island, NY), 10% FBS. CHO cells were transduced with lentiviral particles for each of the CB1 isoforms developed from plasmids containing the appropriate sequence. Vectors for CB1 receptor isoforms were kindly donated by Alex Straiker [13]. Cells were plated on 24-well plates with regular media 24 h before transduction. Transductions were performed with 1 μl of Lentifect™ Lentivirus (Genecopoeia, Rockville, MD) and 8 μg/ml of Polybrene® (Santa

Cruz, Dallas, TX) for 24 h. Selection with 10 μg/ml of Puromycin (Invitrogen) was done 72 h after.

2.3. Gel electrophoresis and Western blot analysis

CHO cells stably transduced with human CB1 and CB1b receptor splice variants were lysed in RIPA buffer containing 1 mM sodium orthovanadate, phosphatase and protease inhibitor cocktails, centrifuged at 12,000g for 15 min at 4 °C, and protein quantified using the bicinchoninic acid assay kit (Pierce, ThermoScientific, MA). Protein extracts were prepared with Laemmli sample buffer containing 2, 3, 5 or 6% of SDS and incubated at 37 °C or 95 °C for 30 or 5 min respectively. Protein extracts were resolved on Novex 4%–12% Tris-glycine gels (Invitrogen) under reducing conditions and transferred to polyvinylidene difluoride membranes using the Trans-Blot® SD Semi-Dry Transfer Cell (BioRAd, CA) for 90 min at 25 V. Membranes were blocked with 5% milk (BioRad) in TBS-T. Rabbit polyclonal antibodies generated against CB1 receptor (Rb-Af380-1; Frontier Science, NY) was used 1:1000 in 5% BSA TBS-T overnight at 4 °C. Peroxidase-conjugated secondary antibody was purchased from GE Healthcare (Piscataway, NJ) and ECL reagents from GE Healthcare.

2.4. In-gel digestion

2.4.1. Protein precipitation—CB1 cells were lysed using 600 μl of lysis buffer Tris [50 mM, pH 8.0] containing 8 M urea, 2 M Thiourea, 150 mM NaCl, 4% CHAPs, 1% Triton X, 100 mM DTT and 2 μl protein protease inhibitor. The cells were lysed by sonication on ice (3 s pulse and 15 s breaks) for 5 min followed by passing the cell suspension through 26.5G gauge needle 5 times. The samples were centrifuged at 16,000 × g (Eppendorf Centrifuge 5415D) for 30 min at 4 °C. The supernatant was collected and treated with 10% (w/w) trichloroacetic acid (TCA) in acetone. The solution was kept at 4 °C (1 h for CB1b; 18 h CB1). The solution was centrifuged at 16,000 × g for 15 min at 4 °C, and the precipitate was washed three times with acetone and dried in speed vac for 5 min.

2.4.2. Protein gel electrophoresis (1D PAGE) and digestion—The precipitate was dissolved in 600 μl of resuspension buffer Tris [50 mM, pH 8.0] containing 8 M urea, 2 M Thiourea, 150 mM NaCl, 100 mM DTT by pipetting at 10 min interval for 30 min at room temperature. The protein concentration was estimated by 2D Quant kit (GE Healthcare). To the solution, 200 μl of Lithium dodecyl sulphate (LDS) sample buffer (Invitrogen) was added and the mixture was warmed at 35 °C for 30 min and kept on ice for 10 min. 600 μg of total protein was loaded in 4–12% "NuPAGE Novex Bis-Tris" 1 mm gel with running buffer as MES buffer. The gel was stained by Silver Stain [18]. After the gel was washed, the protein band was cut into 1cc slices. The gel pieces were destained using 1:1 mixture of 30 mM Potassium ferricyanide and 100 mM Sodium thiosulphates until the gel pieces were no longer brown. They were rinsed with water three times followed by wash with ammonium bicarbonate [25 mM, pH 8] for 10 min. The buffer was discarded and washed with acetonitrile. It was further re-swelled in ammonium bicarbonate [25 mM, pH 8], dehydrated in acetonitrile. The gel pieces were dried in speed vac (savant, SPD111 V) for 20 min. The gel pieces were then incubated in 0.1 M dithiothreitol (DTT) prepared in ammonium bicarbonate [25 mM, pH 8] at 60 °C for 1 h and subsequently, washed with ammonium

bicarbonate [25 mM, pH8], followed by incubation with 500 μl of 55 mM iodoacetamide in ammonium bicarbonate [25 mM, pH8] for 30 min in the dark. The gel pieces were then washed twice with ammonium bicarbonate [25 mM, pH 8] and dehydrated by washing with acetonitrile and dried in a speed vac for 30 min. To the dried gel pieces 100 μl of trypsin (12 ng/μl) (Promega) prepared in ammonium bicarbonate [50 mM, pH 8.0] was added and kept overnight at 37 °C. The peptides were collected in a separate vial. To the gel pieces 300 μl of 60% acetonitrile, 0.1% formic acid followed by 90% acetonitrile, 0.1% formic acid. The solution was evaporated under speed vac. The peptides were dissolved in 500 μl water 0.5% trifluroacetic acid (TFA). To the peptides 1 μl of 500 pg/μl of the internal standards was added. The resulting solution was desalted using Oasis HLB cartridge (Waters, Cat No-094225). The peptides were extracted with 80% acetonitrile 0.1% Formic acid. The elute was dried in a speed vac and reconstituted in 5% acetonitrile 0.1% formic acid. Calibration curves were prepared with spiked standards in native CHO-cells (CB1: 2.18–70 ng/ml and 500 ng/ml for the internal standard; CB1b: 0.185–6 μg/ml and 500 ng/ml for the internal standard). The quality control concentrations were as follows: 7, 25 and 40 ng/ml for CB1 and 1, 2 and 6 μg/ml for CB1b. The quantification of CB1 and CB1b was accomplished using area ratios calculated using the corresponding heavy label as the internal standard.

2.5. Immunoprecipitation

The immunoprecipitation of CB1 and CB1b was carried out following a previously published protocol with slight modifications [19]. Briefly, CHO-CB1 or CHO-CB1b cells were lysed in 100 μl Tris HCl buffer [50 mM, pH 7.4] containing 150 mM NaCl, 10 mM iodoacetamide, 2 mM PMSF, 20 mM Na_3MoO_4, 0.5% NP-40 and protease inhibitor cocktail (SIGMA) for 30 min in ice followed by s sonication bursts 3 times. The solution was centrifuged at 16,000g for 5 min. To the supernatant 2.5 μg Anti-CB1 Polyclonal Antibody (Immuno-Genes, Switzerland) was added and mixed overnight at 4 °C. Then 10 μl slurry of prewashed Protein G agarose beads (Millipore, Billerica, MA) was added and stirred for 2 h at 4 °C. The solution was centrifuged at 500g for 1 min and the supernatant was removed. The beads were washed three times 100 μl of PBS, after which 60 μl of buffer containing 2 M urea, 50 mM ammonium bicarbonate and 5 μg/μl trypsin, 0.1% Protease Max was added for 30 min at 27 °C. The solution was centrifuged for 30 s at 500g, the supernatant was removed and the beads were washed twice with 25 μl of ammonium bicarbonate [50 mM, pH 7.5] containing 2 M urea and 1 mM DTT. The washes and the supernatant were pooled and incubated overnight at room temperature. 20 μl of iodoacetamide (5 mg/ml) was added for 30 min in the dark, then 1 μl of TFA was added. The samples were desalted and reconstituted in 25 μl 95:5 (water: acetonitrile) containing 0.1% formic acid.

2.6. Targeted proteomic analysis

The tryptic peptides for CB1 and CB1b were selected using NCBI BLAST and UniProt/ BLAST searches, with further support for selection of peptides and optimization of transitions through Skyline (Seattle Proteome Center). The CB1 peptide: FPLTSFR and CB1b peptide: TITTDLLGSPFQEK were purchased from New England Peptide (Gardner, MA). For the heavy peptides the C terminal arginine or the lysine was ^{13}C and ^{15}N labelled. The purity of the peptides was greater than 95% as obtained from HPLC. The chromatographic separation was achieved at room temperature on a Waters XBridge C18

column (2.1 mm id × 100 mm, 3.5 μm) protected with a Waters XBridge C18 guard column. The mobile phase consisted of water with 0.1% formic acid as Component A and acetonitrile with 0.1% formic acid as component B. A linear gradient was run as follows: 0 min 5% B; 3 min 5% B; 15 min 15% B; 16 min 15% B; 40 min 50%B; 50 min 80%; 51 min 80%; 53 min 5%B at a flow rate of 0.2 ml/min. The injection volume per sample is 20 μl and the total run time was 60 min per sample. MS/MS analysis was performed using API 5500QTrap system from Applied Biosystems/MDS Sciex equipped with Turbo V electrospray ionization source (TIS)$^®$ (Applied Biosystems, Foster City, CA, USA). The data was acquired and analyzed using Analyst version 1.5.1 (Applied Biosystems). Positive electrospray ionization data were acquired using selected reaction monitoring (MRM). The TIS instrumental source settings for temperature, curtain gas, ion source gas 1 (nebulizer), ion source gas 2 (turbo ion spray) and ion spray voltage were 550 °C, 20 psi, 60 psi, 70 psi and 5500 V, respectively. Declustering potential (DP), collision energy (CE) and collision exit potentials (CXP) were optimized for the standard and heavy peptides (the terminal amino acid is 13C labelled). CB1: FPLTSFR: 434.24/360.69 (116 V,23 V,14 V); 434.24/623.351 (45 V,25 V, 10 V); 434.24/510.26 (45 V,25 V, 10 V); 434.24/720.404 (45 V, 25 V,10 V); heavy (H$_2$N-FPLTSFR-OH) 439.3/520.3 (91 V, 29 V, 16 V); 439.3/365.4 (117 V, 23 V, 16 V); 439.3/119.8 (91 V, 45 V, 16 V); CB1b: TITTDLLGSPFQEK: 775.408/792.389 (86 V, 33 V,36 V); 775.408/648.335 (86 V, 33 V, 36 V); 775.408/905.473 (120 V, 36 V, 10 V); 775.408/1018.57 (43 V, 38 V, 10 V) and heavy (H$_2$N-TITTDLLGSPFQEK-OH) 779.510/800.430 (86 V,33 V,36 V); 779.510/656.381 (86 V, 47 V, 18 V).

3. Results and discussion

Western blotting has been used in characterizing protein expression levels of CB1 and CB2 receptors with mixed results [14]. For example, with endocrine cells in the islets of Langerhans [1,3,5–8,11,12,16,20,21], there are conflicting data regarding the expression of CB1 or CB2 receptors. In addition, post-translation modifications may result in additional bands. CHO cells were transduced with CB1 full length or with CB1b lentiparticles. Protein extracts from CHO-CB1 were incubated with increasing concentrations of SDS (2, 3, 5 or 6%) at 37 °C or 95 °C for 30 or 5 min respectively. After SDS-PAGE, we found that the best conditions for resolving CB1 receptor on a gel electrophoresis were 5% SDS at 37 °C for 30 min. We then performed an SDS-PAGE in those conditions with protein extracts from CHO-CB1 and CHO-CB1b (data not shown). The expected molecular weights are ~53, and 49 KDa for CB1, and CB1b respectively. Multiple bands were observed at various molecular weights. It is important to note that western blot analysis was not able to distinguish the difference between CB1 and its isoforms. This may be due to post-translational modifications that can alter the run of the proteins in the gel such as glycosylation, as well as to the lack of specificity of the antibodies for the isoforms. Therefore, we sought an alternative approach, targeted proteomics, for the characterization of the CB1 and CB1b isoform.

Targeted Proteomics is a quantitative assay for the determination of a targeted protein from a complex matrix using specific peptides. This approach offers advantages over western blot analysis as no antibodies are required and consequently overcomes the limitations of the

antibody recognition of highly similar homologous proteins. For CB1 and CB1b receptor, the redundancy in the primary amino acid sequence is greater than 93% [10], with amino acids 22–54 excised in the CB1b isoform. As a result of the high homology, while typically a minimum of three peptides is preferred for a targeted proteomic approach, in this case, only one peptide sequence can be used for each protein (Fig. 1). *In silico* digestion of the CB1 and CB1b was performed using Protein prospector (University of California, San Francisco). Each peptide for CB1 and CB1b was synthesized to determine the presence of CB1 and CB1b proteins in a cellular matrix. The target peptides: FPLTSFR and TITTDLLGSPFQEK were synthesized for the characterization of the CB1 and CB1b receptors, respectively. For both peptides, four transitions were optimized by MS to achieve the greatest sensitivity. Optimized MS parameters for the respective peptides, with Q1 and Q3, declustering potential (DP), collision energy (CE), entrance potential (EP), and collision cell exit potential (CXP) are reported in Table 1. HPLC optimization results showed a good resolution of peptides (Fig. 2A–B) with a total run time of 60 min using a gradient method with retention times of 25.4 min for CB1 and 27.5 for CB1b.

The sample preparation for the transduced CHO cells was optimized in order to enrich the amount of isolated CB1 and CB1b receptors. While several methods exist including gel electrophoresis (1D and 2D), immunoprecipitation, or abundant protein depletion strategy, in this study, only in gel digestion and immunoprecipitation were carried out. Both the transduced and native CHO cells were lysed by sonication followed by passing the cell suspension through needle. Lysis was followed by protein precipitation and resuspension [22], where it was determined that 1 h at 4 °C was sufficient for the CB1b isoform, but 18 h at 4 °C was necessary to have quantitative levels for the CB1 receptor. For protein resuspension, both RIPA and Tris buffer containing urea were used and it was determined that Tris buffer with urea was more effective. While typically, the denaturation step for in-gel digestion is carried out for 10 min at 70 °C, these conditions resulted in non-detectable levels for either CB1 and/or CB1b. As a result, multiple temperatures were assessed and it was determined that 35 °C for 30 min was optimal. For the in-gel digestion of CHO-CB1 cells, only the transitions for CB1 were observed, while for the CHO-CB1b cells, only the CB1b transitions were observed (Fig. 3A–B). While optimizations were carried out for immunoprecipitation [14], the required concentration of the primary antibody was cost-prohibitive and, as a result, the in gel digestion method was preferred.

While, in most SRM assays, the addition of the internal standard is typically done prior to analysis, in this study, the internal standard was added at various stages of the sample preparation including pre- and post-desalting and prior to injection with spiked CB1 standard in native CHO cells. A 30% decrease in the calculated concentration was observed when comparing the addition of the internal standard prior to desalting versus prior to analysis (0.30 pg/μg vs 0.21 pg/μg respectively). Further, the addition of the internal standard after desalting resulted in 10% change in calculated concentration of the peptide (0.30 pg/μg vs 0.27 pg/μg, respectively). The results of this study indicate that the addition of the internal standard prior to desalting is necessary and that addition at later stages may result in decreased reported values. The targeted proteomic approach was developed for both peptides in native CHO cells using the 5500 QTRAP, no significant matrix effects were

observed for these peptides. The top 3 transitions for both the target peptide of CB1 and in CB1b were observed in the CHO cells transduced with CB1 and CB1b respectively.

The calibration curves were found to be linear over the range of 2.18–70 ng/ml for CB1 ($r^2 >$ 0.999) and 0.37–6 μg/ml for CB1b ($r^2 > 0.994$). Quality control (QC) (7, 25, 40) ng/ml for CB1 and (1,2,6) μg/ml for CB1b were spiked in native non-transduced CHO cells and intra and interday accuracy and precision were determined (Table 2). Intraday precision (CV) ranged from 1.9% to 4.3%, and inter-day precision (CV) ranged from 1.2% to 5.2% for CB1. In case of CB1b, the intraday precision ranged from 0.4% to 5.9% while interday precision ranged from 1.2% to 6.1%. The intraday accuracy ranged from 89.7% to 109.8% while inter-day accuracy ranged from 90.6% to 110.3% for CB1. The intraday and interday accuracy for CB1b ranged from 92.01% to 118.5% and 95.2% to 119.05% respectively.

The SRM method was applied to determine the concentration of CB1 and CB1b in CHO cells transduced with the CB1 and the CB1b isoform. Based on the developed SRM assay, the concentration of CB1 and CB1b in CHO-CB1 cells and CHO-CB1b cells was determined to be 7.15 ng/ml and 0.87 μg/ml respectively, which fell in the linear range of the developed method. The concentration was further normalized to the total protein content and was determined to be 192.07attomol/μg and 23.39 fmol/μg for target peptide of CB1 and CB1b respectively.

A targeted proteomic SRM assay was developed for the determination of CB1 and CB1b in a cellular matrix. All QCs had precision less than <6% and accuracy within 20% of the nominal concentration. The developed assay may be used in determination of CB1 and CB1b in other matrices including tissue.

Acknowledgments

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CB1 :   ------------------------------------------MKSILDGLADTTFRTITTDLLY 22
CB1b:   -------------------------------------------MKSILDGLADTTFRTITTDLL- 21


CB1 :   VGSNDIQYEDIKGDMASKLGYFPQKFPLTSFRGSPFQEKMTAGDNPQLVP 72
CB1b:   ----------------------------------------GSPFQEKMTAGDNPQLVP 39


CB1 :   ADQVNITEFYNKSLSSFKENEENIQCGENFMDIECFMVLNPSQQLAIAVL 122
CB1b:   ADQVNITEFYNKSLSSFKENEENIQCGENFMDIECFMVLNPSQQLAIAVL 89
```
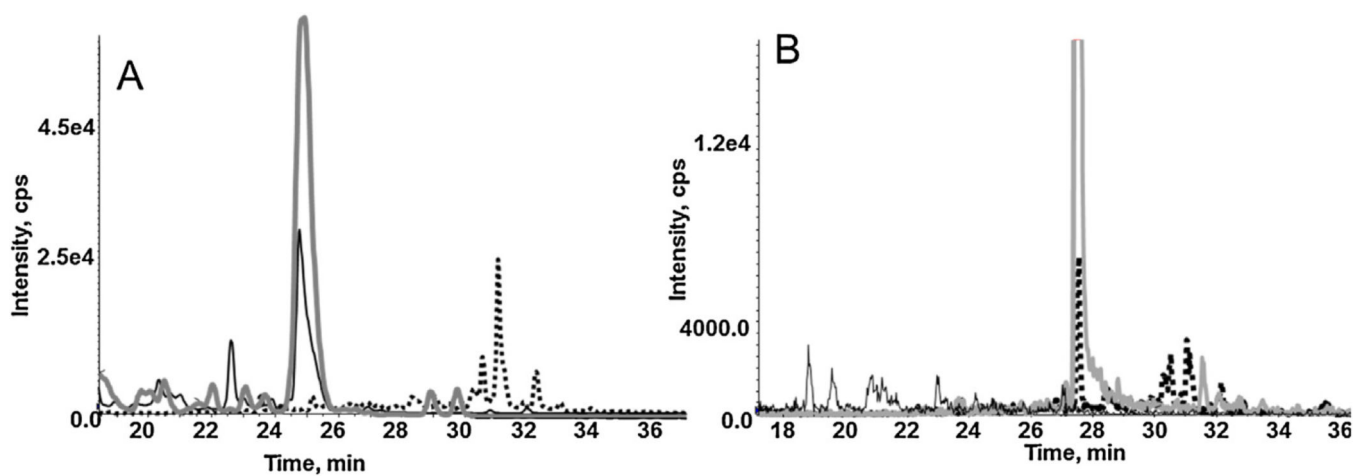
Fig. 1.
The amino acid sequence of the CB1 and CB1b. The target peptide chosen for SRM analysis for CB1 and CB1b is in bold.
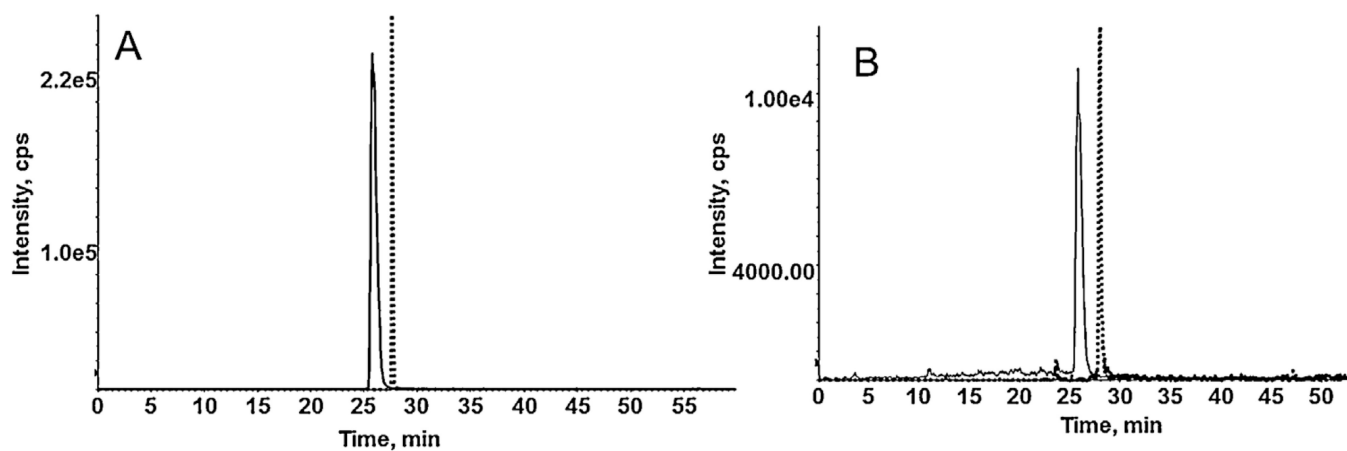
Fig. 2.
Chromatogram of CB1 (FPLTSFR) (434.2/360.6, solid line) and CB1b
(TITTDLLGPFQEK) (775.40/792.38, dashed line) in 95:5 (water: acetonitrile, containing
0.1% formic acid) (A) and spiked in native CHO cells digested by in-gel digestion method
(B).

Fig. 3.
Chromatogram of CB1 (FPLTSFR) (434.2/360.6, solid line) and CB1b
(TITTDLLGPFQEK) (775.40/792.38, dashed line) and their respective internal standards
(grey line) in CHO-CB1 (A) and CHO-CB1b (B).

Table 1

Parameters for SRM transitions for FPLTSFR and TITTDLLGSPFQEK. Q1, Q3, DP, CE, CXP for the different transitions for both the standards and internal standards are enlisted.

	Q1	Q3	DP(V)	CE(V)	CXP(V)
FPLTSFR	434.24	360.69	116	23	14
	434.24	623.351	45	25	10
	434.24	510.26	45	25	10
	434.24	720.40	45	25	10
FPLTSFR (HEAVY PEPTIDE)	439.3	520.3	91	29	16
	439.3	365.4	117	23	16
TITTDLLGSPFQEK	775.408	792.389	86	33	36
	775.408	648.335	86	33	36
	775.408	905.473	120	36	10
	775.408	1018.57	43	38	10
TITTDLLGSPFQEK (HEAVY PEPTIDE)	779.510	800.430	86	33	36
	779.510	656.381	86	47	18

Table 2

Intraday and interday precision and accuracy for CB1 and CB1b in native CHO cells.

	Concentration CB1 (ng/ml)			Concentration CB1b (µg/ml)		
	7	**25**	**40**	**1**	**2**	**6**
Intra-day CV (%)	1.9	3.3	4.3	1.1	0.4	5.9
Inter day CV (%)	5.2	1.2	2.8	1.9	1.2	6.1
Intra-day Accuracy	109.8	89.7	91.9	114.1	118.5	92.01
Inter-day Accuracy	110.3	90.6	90.8	115.7	119.05	95.28