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# Quantification of new antiepileptic drugs by liquid chromatography/electrospray ionization tandem mass spectrometry and its application to cellular uptake experiment using human placental choriocarcinoma BeWo cells

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**Abbreviations used:** GBP, gabapentin; IS, internal standard; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LEV, levetiracetam; LLOQ, lower limit of quantification; LTG, lamotrigine; R.E., relative error; R.S.D., relative standard deviation; SRM, selected reaction monitoring; TPM, topiramate

**Abstract**

A method for quantification of new antiepileptic drugs, including lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM), in cellular samples, using liquid chromatography/electrospray ionization tandem mass spectrometry was developed to better understand the membrane transport mechanisms of these drugs. Cell lysate was deproteinized by methanol containing LEV-$d_3$ as an internal standard (IS). Chromatographic separation was performed on a C18 column using gradient elution with methanol–water–formic acid (10:90:0.1, v/v/v) and methanol–formic acid (100:0.1, v/v). Analytes were detected in positive ion electrospray mode with selected reaction monitoring (SRM). This method was applicable for a linear range of 5 to 500 pmol for LTG; 5 to 1000 pmol for LEV; 10 to 10,000 pmol for GBP; and 5 to 5,000 pmol for TPM. The intra-day precision, inter-day precision, and accuracy data were assessed and found to be acceptable. This developed and validated method was then successfully applied to the investigation of uptake of the new antiepileptic drugs in placental choriocarcinoma BeWo cells. The intracellular concentration of these drugs in BeWo cells, accumulating over 30 min at 37°C was in the order of GBP>LTG>LEV≈TPM. Furthermore, the uptake of GBP at 4°C was much lower than that at 37°C. The uptake of GBP was saturated at high concentrations. The kinetic parameters calculated for GBP uptake in BeWo cells were determined as *K*m of 105.4 ± 6.4 µM and *V*max at 8153 ± 348 pmol/mg protein/min. The novel method described here should enable investigators to elucidate the transport mechanisms of these antiepileptic drugs in BeWo cells.

**Keywords:** cellular uptake experiment; gabapentin; lamotrigine; levetiracetam; liquid chromatography/tandem mass spectrometry; topiramate

## 1. Introduction

Exposure to some antiepileptic drugs during pregnancy is associated with an increased risk to the fetus. For example, valproic acid (VPA), which belongs to an earlier generation of antiepileptic drugs, is known to be teratogenic [1]. Furthermore, several studies have indicated that VPA is associated with neurodevelopmental delay [2]. Newer antiepileptic drugs, such as lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM), which became available in the 1990s, are being used with increasing frequency, as per the Australian Pregnancy Register [3]. Most women with epilepsy continue their medication during pregnancy. Understanding the process of placental transfer of antiepileptic drugs at each stage of gestation is important [4]. However, little information is currently available on the detailed mechanisms underlying the placental transfer of new antiepileptic drugs.

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is widely used in the quantification of various drugs and endogenous substances owing to its high sensitivity, specificity, and capability for simultaneous analyses. Kim *et al*. reported an LC/MS/MS method for the quantification of ten antiepileptic drugs in human plasma [5]. Shibata *et al*. have developed a method for the quantification of 22 antiepileptic drugs in human plasma by UPLC/MS/MS [6]. Recently, Deeb *et al*. described an LC/MS/MS method for the quantification of 22 antiepileptic drugs in postmortem blood, serum, and plasma [7]. However, few methods have been developed for quantifying antiepileptic drugs at the cellular level.

Membrane transporters play a role in the pharmacokinetics and physiological processes in several organs, including placenta. To prevent potential drug-drug interactions and consider individual variation, gathering information on the role of drug transporters in pharmacokinetics and pathophysiological processes is important. However, the mechanism of uptake of new antiepileptic drugs by placental cells and the contribution of drug transporters to placental transfer during the entire pregnancy have not yet been fully elucidated. For this purpose, we developed a simple and robust LS/MS/MS based method for the quantification of major new antiepileptic drugs, including LTG, LEV, GBP, and TPM, in cell extracts. In this study, we describe our novel method and its application to human placental choriocarcinoma BeWo cells to measure drug uptake.

## 2. Materials and methods

### 2.1 Chemicals

LTG (purity ≥98%), LEV (purity ≥98%), GBP (purity ≥98%), and TPM (purity ≥98%) were purchased from Tokyo Chemical Industry (Tokyo, Japan). LEV-$d_3$ was purchased from Toronto Research Chemicals (North York ON, Canada). HPLC-grade methanol and formic acid were purchased from Wako (Tokyo, Japan).

### 2.2 Standards and calibration curve preparation for LC/MS/MS analysis

Standard stock solutions containing mixtures of LTG, LEV, GBP, and TPM were prepared in

methanol (0.5, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 µM). An internal standard (IS) stock solution containing LEV-$d_3$ (50 ng/mL) was also prepared in methanol. All stock solutions were stored at −80°C. Calibration solutions were prepared from stock solutions in 0.3 mL of blank cell lysate sample.

**2.3 LC/MS/MS**

HPLC was performed using a completely equipped Prominence 20A (Shimadzu, Kyoto, Japan) system. The separation was performed on an Inertsil ODS-3 column (150 × 2.1 mm i.d., 5 µm, GL Science Inc., Tokyo, Japan) and elution was performed on a gradient. The mobile phase flow rate was set at 0.25 mL/min. Mobile phase A consisted of methanol/water/formic acid (10:90:0.1, v/v/v), and mobile phase B consisted of methanol/formic acid (100:0.1, v/v). Mobile phase B was increased from 0% to 80% in a linear gradient over 2 min and maintained at 80% for the first 4 min. Mobile phase B was then decreased to 0% from 4 min to 5 min and maintained at 0% until 10 min. From 3 to 7 min, the flow was introduced into a mass spectrometer using a switching valve. The column temperature was maintained at 40°C. The injection volume was 10 µL and the overall run time was 10 min. Mass spectrometry was carried out using an Applied Biosystems (Foster City, CA) API 3200™ LC/MS/MS System. The mass spectrometer was operated in positive ion electrospray mode with selected reaction monitoring (SRM). SRM transitions monitored were *m/z* 256 → 211 for TLG, *m/z* 171 → 126 for LEV, *m/z* 172 → 137 for GBP, *m/z* 340 → 264 for TPM, and *m/z* 174 → 129 for LEV-$d_3$ (IS). The parameter settings were as follows: source temperature of 250°C, spray voltage of 5500 V, curtain gas of 50 psi, ion source gas 1 of 30 psi, ion source gas 2 of 30 psi, collision gas of 5 arbitrary units, and dwell time of 200 ms per ion. Data were acquired and analyzed using Analyst software (version 1.5) (Applied Biosystems).

**2.4 Cell culture**

BeWo cells were obtained from the Riken Cell Bank (Saitama, Japan). BeWo cells were kept in the nutrient mixture F-12 Ham Kaighn's modification (Wako) supplemented with 15% fetal bovine serum (Thermo scientific, Rockford, IL) and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO) at 37°C under 5% $CO_2$. For the uptake experiment, BeWo cells ($5 \times 10^4$ cells/well) were seeded on 24-well collagen-coated plastic plates. After the cells had grown to confluence, they were used for the uptake experiment.

**2.5 Uptake experiment**

After removal of the growth medium, cells were washed with transport buffer and pre-incubated at 37°C for 10 min with 0.5 mL of transport buffer. Transport buffer consisted of Hank's balanced salt solution (HBSS) with 25 mM HEPES at pH 7.4. Uptake was initiated by applying 0.5 mL of transport buffer containing the drugs (50 µM) under study. After incubation, cells were immediately rinsed three times with ice-cold transport buffer. The cells were lysed with 200 µL

of 1 N NaOH. After confirmation of cell lysis by observing with a microscope, the lysate was neutralized with 100 µL of 2 N HCl. The protein concentration was determined using a Pierce® BCA Protein Assay Kit (Thermo scientific), in accordance with the manufacturer's instructions.

### 2.6 Sample preparation

To 100 µL of cell lysate, 100 µL of methanol containing IS (50 ng/mL) was added. After vortexing, the samples were centrifuged for 5 min at 13,000 ×*g*, and 10 µL of the supernatant was injected into the LC/MS/MS.

### 2.7 Method validation

#### 2.7.1 Linearity and lower limit of quantification (LLOQ)

Calibration solutions were freshly prepared from stock solutions in 300 µL of blank cell lysate by spiking with 10 µL of standard stock solutions at concentrations of 5, 10, 50, 100, 250, and 500 pmol for LTG, 5, 10, 50, 100, 250, 500, and 1000 for LEV, 10, 50, 100, 250, 500, 1000, 2500, 5000, and 10,000 pmol for GBP, and 5, 10, 50, 100, 250, 500, 1000, 2500, and 5000 pmol for TPM. The samples were prepared as described in *Sample preparation* and analyzed. Calibration curves were constructed by plotting the peak area ratio (standard to IS) versus the nominal concentration and were fitted using least-squares regression with 1/x weighting. LLOQ was defined as the concentration with a signal-to-noise (S/N) ratio of at least 10 and acceptable precision and accuracy data [relative standard deviation (R.S.D.) and relative error (R.E.) less than 20%].

#### 2.7.2 Precision and accuracy

Intra-day precision and accuracy were assessed by measuring six samples at four concentrations (5, 50, 100 and 500 pmol for LTG, 5, 50, 500 and 1000 pmol for LEV, 10, 100, 1000 and 10000 pmol for GBP, and 5, 50, 500 and 5000 pmol for TPM) on the same day. Inter-day precision and accuracy were assessed by measuring the samples at four concentrations on six different days. The samples were prepared as described in *Sample preparation* and analyzed. The R.E. (%) was calculated as [(observed concentration − theoretical concentration)/theoretical concentration] × 100 (%). The precision was obtained as the relative standard deviation (R.S.D.).

#### 2.7.3 Recovery and matrix effect

Recovery was assessed by comparing the peak areas of analytes spiked before sample preparation with those of analytes spiked after sample preparation. Matrix effect was assessed by comparing the peak areas of analytes spiked after sample preparation with those of an equivalent amount of analytes prepared in mobile phase A.

#### 2.7.4 Stability

The stability of the analytes, both short-term and under freeze-thaw conditions, were

examined by measuring the amounts remaining after the addition of analytes to the blank cell lysate and subjecting it to one of the following conditions: for evaluation of short-term stability, the samples were stored at room temperature for 6 h prior to measurement. Freeze-thaw stability was assessed following three freeze-thaw cycles (−80°C to room temperature).

**2.8 Statistical analysis and kinetic parameters**

Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance was defined as $p < 0.05$.

Nonlinear regression analysis was performed by using Origin® (version 9.1J) (OriginLab Corporation, MA). Kinetic parameters were obtained using the following equation:

$v = Vmax \times s / (Km + s)$

where v is the uptake rate of compounds, s is the compound concentration, *K*m is the Michaelis–Menten constant, and *V*max is the maximum uptake rate.

## 3. Results and discussion

### 3.1 Method optimization

A gradient elution with methanol/water/formic acid and methanol/formic acid, described in *Materials and Methods*, produced optimal separation with optimal sensitivity. Representative chromatograms are shown in Fig. 1. The retention times of LTG, LEV, GBP, and TPM were 4.2, 4.4, 3.9, and 5.2 min, respectively.

Sample treatment to solubilize cells after completing the uptake experiment was performed based on the method described by Wang *et al* [8]. Cells were lysed with NaOH and subsequently neutralized with HCl. Because LTG, GBP, and TPM were stable in 1N NaOH for at least 30 min (nearly 100%) and LEV was stable within 15 min (> 90%) (data not shown), the cells were treated with 1N NaOH with 15 min. After confirmation of cell lysis, the lysate was neutralized with HCl.

### 3.2 Validation

#### 3.2.1 Calibration curve

Calibration curves were linear in the range of 5 to 500 pmol for LTG, 5 to 1,000 pmol for LEV, 10 to 10,000 pmol for GBP, and 5 to 5,000 pmol for TPM. Typical standard curves were $y = 0.00142x + 0.00151$ ($r = 0.9972$) for LTG, $y = 0.0185x + 0.021$ ($r = 0.9998$) for LEV, $y = 0.00447x - 0.0116$ ($r = 0.9987$) for GBP, and $y = 0.00284x - 0.000227$ ($r = 0.9999$) for TPM.

#### 3.2.2 Specificity and selectivity

Fig. 1 shows the representative chromatograms of blank cell lysate (A), blank cell lysate with IS and LLOQ levels of analytes (B), and cell sample collected after the uptake experiment (C). As shown in Fig.1 (A), a significant interference was not observed in the blank cell sample at the time of retention for LTG, LEV, GBP, and TPM, showing that the method has optimal specificity.

#### 3.2.3 Precision and accuracy

The intra-day precision ranged from 2.9% to 13%, and the accuracies ranged from −7.6% to 18.3% (Table 1). The inter-day precision ranged from 2.2% to 16.6%, and the accuracies ranged from −11.4% to 15.7%. The precision and accuracy were within 15%, except for LLOQ (those of LLOQ were within 20%).

#### 3.2.4 Matrix effect and recovery

The extraction recovery of analytes ranged from 87.4% to 116.9% (Table 2). As a sample pretreatment, cell lysate was deproteinized by methanol containing IS. Matrix effects (% ionization suppression) ranged from 0.6% to 29.1%. Although the matrix effects could not be completely eliminated, we selected the present method for its precision and data accuracy.

#### 3.2.5 Stability

For evaluation of short-term stability, the amount of drug remaining following storage for 6 hours at room temperature was measured (Table 3). No significant degradation was observed for any of the drugs (92.3%–102.3%). Freeze-thaw stability was assessed following three freeze-thaw cycles (−80°C to room temperature); no significant degradation was observed (90.4%–112.7%).

### 3.3 Determining uptake of drugs by human placental choriocarcinoma BeWo cells

We applied the above-characterized method to a cellular uptake experiment using human placental choriocarcinoma BeWo cells. As shown in Fig. 2 (A), the cellular accumulation after incubation for 30 min with 50 μM antiepileptic drugs at 37°C was GBP>LTG>LEV≈TPM. The concentration of 50 μM is near the therapeutic blood levels of these antiepileptic drugs [7]. Fig. 1 (C) shows representative chromatograms of cell samples collected after completing the uptake experiment. Although GBP is hydrophilic under physiological conditions, the accumulation of GBP in BeWo cells was considerably higher than the other drugs. Since the intracellular accumulation of GBP was higher than that of LTG, LEV and TPM, the uptake properties of GBP in BeWo cells were further investigated. As shown in Fig. 2 (B), accumulation of GBP at 4°C was markedly lower than that at 37°C. Fig. 2 (C) shows the time-dependent uptake of GBP by BeWo cells following an incubation with 5 μM GBP. The uptake of GBP is linear up to 10 min, so the initial uptake was evaluated for 5 min. Fig. 2 (D) shows the concentration-dependent uptake of GBP by BeWo cells. The transport of GBP in BeWo cells showed saturation at high concentrations. Eadie–Hofstee plots (*inset* in Fig. 2 (D)) showed a single straight line. The results suggest that a single transporter is involved in the uptake of GBP by BeWo cells. Kinetic parameters *K*m and *V*max were estimated to 105.4 ± 6.4 μM and 8153 ± 348 pmol/mg protein/min, respectively. The *K*m value for GBP uptake was within a physiological range, indicating that a carrier-mediated transport mechanism may play a role in the placental transfer of GBP. Ohman *et al*. have suggested an active mode of placental transport of GBP, based on umbilical-to-maternal GBP plasma concentration ratios ranging from 1.3 to 2.1 (mean, 1.7) [9]. LAT1, a sodium-independent neutral amino acid transporter, has also been shown to be involved in the transport of GBP in brain endothelial cells [10]. The organic cation transporter OCTN1 also contributes to the urinary excretion of GBP [11]. Future studies should investigate the role of these transporters in placental cells.

## 4. Conclusion

Here, we report our characterization of a method for the quantification of new antiepileptic drugs, including LTG, LEV, GBP, and TPM, in cellular samples using LC/MS/MS. The method was validated, and found to be reliable with acceptable accuracy and precision. The newly developed and validated method was then employed for the quantification of intracellular LTG, LEV, GBP, and TPM accumulation in the human placental cell line BeWo. To the best of our knowledge, LC/MS/MS methods have not been previously reported for use in the quantification of new antiepileptic drugs in cultured cells. The method we have described here will be useful for future *in vitro* studies of the role of transporters in the placental transfer of LTG, LEV, GBP, and TPM.

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**Figure captions**

**Figure 1.** Representative SRM chromatograms of lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM). (A) Blank cell lysate. (B) Blank cell lysate with LLOQ levels of LTG, LEV, GBP, and TPM. (C) Cell samples obtained after uptake experiment (treatment of 50 μM of each drug for 30 min at 37°C).

**Figure 2.** Determining uptake of drugs by human placental choriocarcinoma BeWo cells. (A) Accumulation of lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM) in BeWo cells. BeWo cells were incubated with transport buffer containing 50 μM of LTG, LEV, GBP, or TPM for 30 min at 37°C. Each column represents the mean ± S.E. of three independent experiments. (B) Temperature dependence of GBP uptake. BeWo cells were incubated with transport buffer containing 5 μM of GBP for 30 min at 37°C or 4°C. Each point represents the mean ± S.E. of three independent experiments. *****; significantly different from 37°C at $p < 0.05$. (C) Time dependence of GBP uptake. BeWo cells were incubated with transport buffer containing 5 μM of GBP at 37°C. Each point represents the mean ± S.E. of three independent experiments. (D) Concentration dependence of GBP uptake. BeWo cells were incubated with transport buffer containing 2.5–500 μM of GBP for 5 min at 37°C or 4°C. The GBP uptake was obtained by subtracting the uptake at 4°C from that at 37°C. Each point represents the mean ± S.E. of three independent experiments. The *inset* shows an Eadie–Hofstee plot of GBP uptake.

**Table 1.** Intra-day and inter-day precision and accuracy of the method for quantification of lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM) in BeWo cell lysate

| | | Intra-day (n=6) | | | Inter-day (n=6) | | |
|---|---|---|---|---|---|---|---|
| | Spiked (pmol/cell lysate) | Found (pmol) | R.S.D. (%) | R.E. (%) | Found (pmol) | R.S.D. (%) | R.E. (%) |
| LTG | 5 | 4.89 | 11.9 | -2.3 | 4.43 | 10.3 | -11.4 |
| | 50 | 52.7 | 7.8 | 5.3 | 52.9 | 7.2 | 5.8 |
| | 100 | 106.2 | 5.6 | 6.2 | 105.7 | 7.4 | 5.7 |
| | 500 | 474 | 2.9 | -5.2 | 483 | 4.2 | -3.4 |
| LEV | 5 | 5.27 | 6.2 | 5.5 | 4.94 | 7.9 | -1.2 |
| | 50 | 50.0 | 8.2 | -0.1 | 50.7 | 4.4 | 1.3 |
| | 500 | 514 | 4.6 | 2.8 | 496 | 2.4 | -0.8 |
| | 1000 | 1031 | 8.1 | 3.1 | 998 | 2.2 | -0.2 |
| GBP | 10 | 11.83 | 6.4 | 18.3 | 11.57 | 16.6 | 15.7 |
| | 100 | 92.4 | 6.4 | -7.6 | 94.9 | 4.2 | -5.1 |
| | 1000 | 962 | 10.4 | -3.8 | 1018 | 12.3 | 1.8 |
| | 10000 | 10397 | 9.3 | 4.0 | 10200 | 6.4 | 2.0 |
| TPM | 5 | 5.71 | 13.0 | 14.3 | 4.67 | 15.1 | -6.6 |
| | 50 | 48.4 | 3.9 | -3.2 | 49.5 | 5.9 | -1.1 |
| | 500 | 503 | 3.3 | 0.7 | 522 | 6.4 | 4.3 |
| | 5000 | 4985 | 4.3 | -0.3 | 4895 | 2.5 | -2.1 |

R.S.D., relative standard deviation R.E., relative error

**Table 2.** The extraction recovery and matrix effect for the quantification of lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM) in BeWo cell lysate

| | Spiked (pmol) | Recovery (%) | Matrix effect[a] (%) |
|---|---|---|---|
| LTG | 10 | 88.8± 3.2 | 99.4 ± 6.1 |
| | 500 | 97.7 ± 1.4 | 96.3 ± 3.3 |
| LEV | 10 | 87.4 ± 5.3 | 76.8 ± 2.8 |
| | 500 | 90.2 ± 2.8 | 70.9 ± 2.3 |
| GBP | 500 | 109.6 ± 4.6 | 84.6 ± 6.0 |
| | 5000 | 116.9 ± 5.1 | 78.1 ± 3.7 |
| TPM | 500 | 91.6 ± 5.6 | 72.6 ± 1.7 |
| | 5000 | 94.3 ± 7.7 | 78.2 ± 1.6 |

Each value represents the mean ± S.D. (n=3)

[a]Matrix effect (%) = − × 100

where A is the peak area of the standard solution and B is the peak area of the analyte spiked after sample preparation. A value of <100% indicates ionization suppression, and a value of >100% indicates ionization enhancement.

**Table 3.** Stability of lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM) in BeWo cell lysate

| | | Stability (%) | |
|---|---|---|---|
| | Spiked (pmol/cell lysate) | Short-term stability (6 h, room temperature) | Freeze-thaw stability (3 cycles, -80°C to room temperature) |
| LTG | 10 | 93.9 ± 12.4 | 95.7 ± 1.8 |
| | 500 | 102.3 ± 3.1 | 97.5 ± 1.0 |
| LEV | 10 | 98.4 ± 4.0 | 112.7 ± 8.1 |
| | 500 | 102.3 ± 3.4 | 108.8 ± 2.2 |
| GBP | 50 | 94.9 ± 3.1 | 90.4 ± 2.3 |
| | 5000 | 93.1 ± 3.2 | 100.9 ± 5.2 |
| TPM | 50 | 94.9 ± 6.4 | 97.9 ± 9.5 |
| | 5000 | 92.3 ± 2.4 | 99.5 ± 6.5 |

Each value represents the mean ± S.D. (n=3)

## Highlights

・An LC/MS/MS method for quantification of LTG, LEV, GBP, and TPM in cells was developed.

・The method was successfully applied to cellular uptake experiment using BeWo cells.

・The intracellular accumulation in BeWo cells was GBP>LTG>LEV≈TPM.

・The uptake of GBP in BeWo cells was saturated at high concentrations.

**Figure 1**

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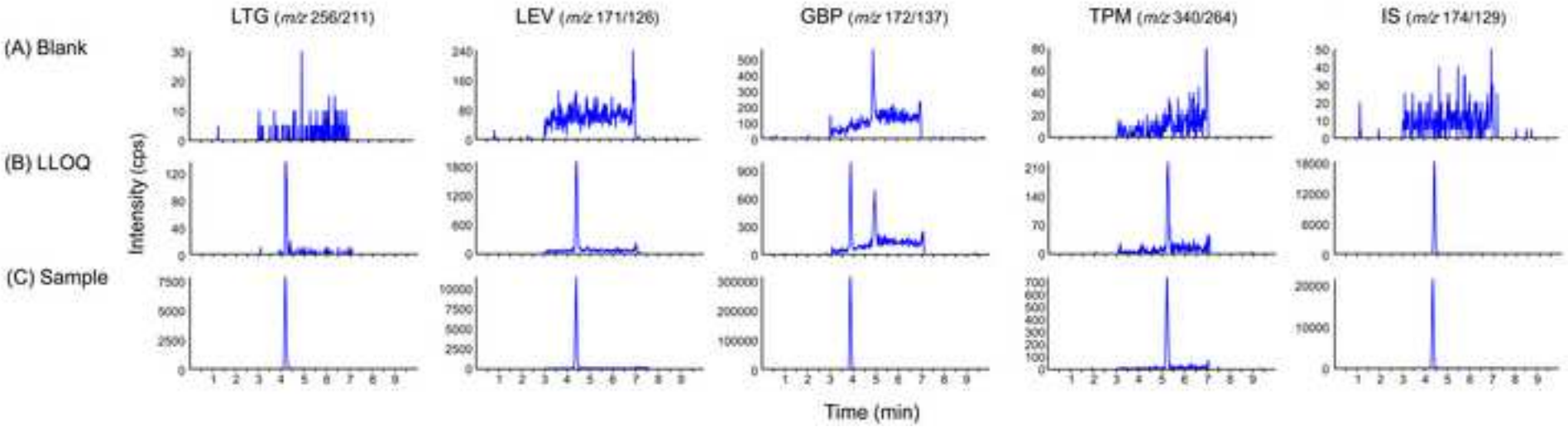
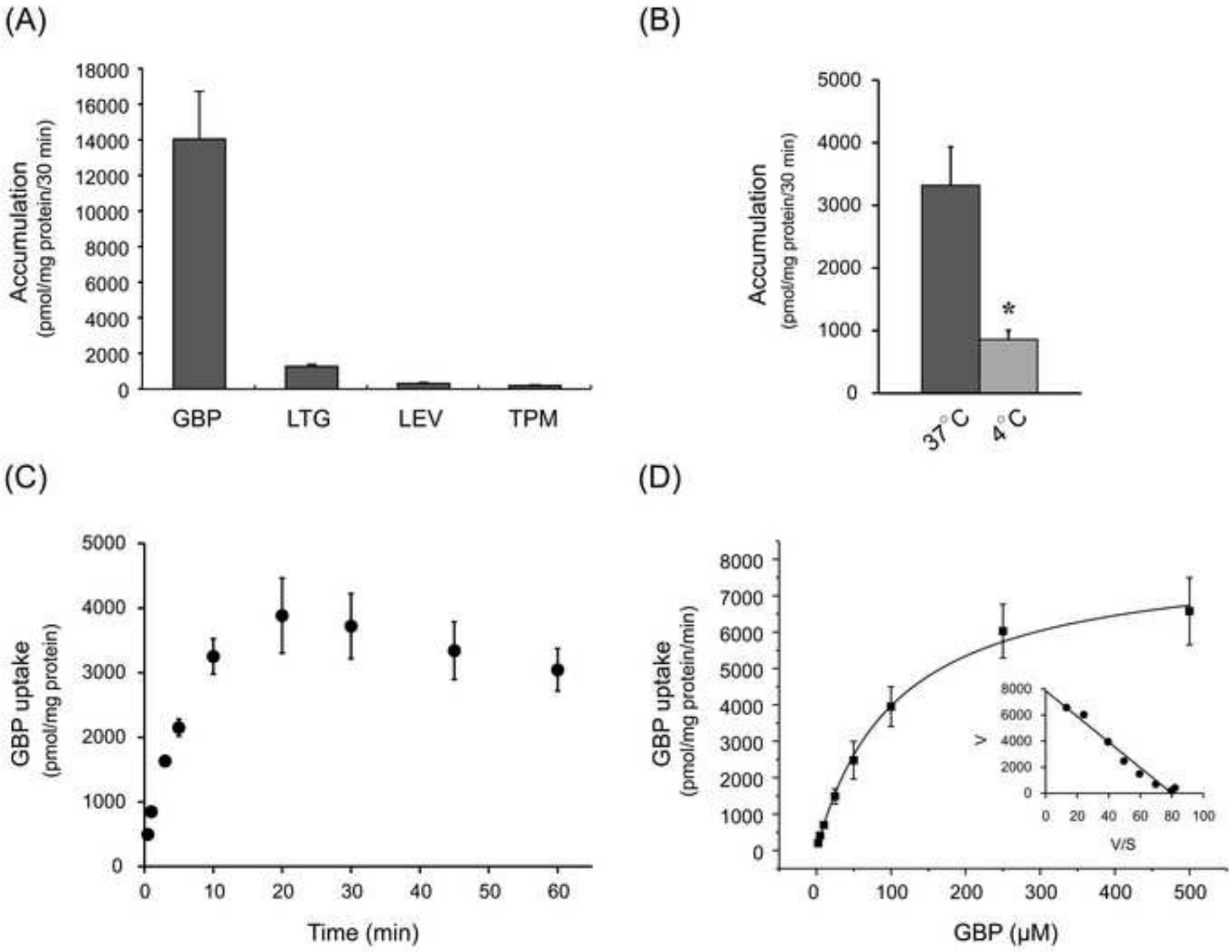


Figure 2

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