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Simultaneous measurement of tryptophan and related compounds by liquid chromatography/electrospray ionization tandem mass spectrometry

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ABSTRACT

We have expanded a liquid chromatographic–tandem mass spectrometric method that measures 3-hydroxykynurenine and 3-hydroxyanthranilic acid in addition to tryptophan and kynurenine both intra- and extracellularly. After reversed phase HPLC separation, the compounds were detected in the MS positive multiple reaction monitoring mode. We found a good linear response for each tryptophan metabolite. The lower limit of quantification for each compound ranged from 0.01 to 0.1 μM. The extraction efficiencies from spiked cell samples and culture medium ranged between 83 and 111% and the overall coefficient of variation of analyses was less than 7%. Using our method, we found tryptophan metabolites in the cells and the culture medium of LN229 human glioma cells were stimulated by interferon-γ, a known inducer of indoleamine 2,3-dioxygenase. The intracellular concentrations of kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid were higher than those in the medium. This is the first report of a method for the simultaneous determination of tryptophan and its metabolic products both intra- and extracellularly.

1. Introduction

The mammalian kynurenine pathway is the major catabolic route for tryptophan [1,2] and is activated during inflammatory processes such as immune activation and neurodegenerative disorders [1,2]. Activation of the pathway decreases the level of tryptophan and increases the concentration of downstream metabolites, including kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid [3,4]. The activation of the kynurenine pathway is thought to induce cell toxicity. Although the decrease in the level of tryptophan has been proposed to trigger cell cytotoxicity, tryptophan metabolites such as kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid are reported individually to inhibit cell proliferation [5,6] and to induce apoptosis [7,8]. Thus, the precise mechanism by which activation of the kynurenine pathway during the inflammatory process induces cell cytotoxicity remains uncertain. Development of a procedure to simultaneously determine the concentration of the various compounds should help to resolve this issue. Currently, it is only possible to measure the levels of tryptophan and its metabolites using separate methods [9–11,14–17]. Such measurements require many biological samples and is a time consuming process. Therefore, it would be a great advantage to simultaneously quantify the concentrations of the compounds using the same analytical procedure. Moreover, although the level of the various compounds in serum have been determined [12,13] the enzymes involved in the kynurenine pathway are intracellular. Therefore, there is a requirement to measure the intracellular concentration of each compound.

A liquid chromatographic–tandem mass spectrometer employing electrospray ionization (ESI) source is a powerful tool that can specifically, sensitively and simultaneously identify and quantify many biological compounds. Bergquist and co-workers recently reported the quantification method for tryptophan and kynurenine in human plasma using capillary liquid chromatography–ESI tandem mass spectrometry (LC–ESI/MS/MS) [14,15]. The aim of the present study was to expand the method to measure 3-hydroxykynurenine and 3-hydroxyanthranilic acid together with tryptophan and kynurenine in both extracellular and intracellular fluids using LC–ESI/MS/MS.

2. Experimental

2.1. Materials

L-Tryptophan, L-kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and L-tryptophan methyl ester were purchased from Sigma–Aldrich Corp. (St Louis, MO). Ammonium formate was purchased from Wako Pure Chemical Industries (Osaka,

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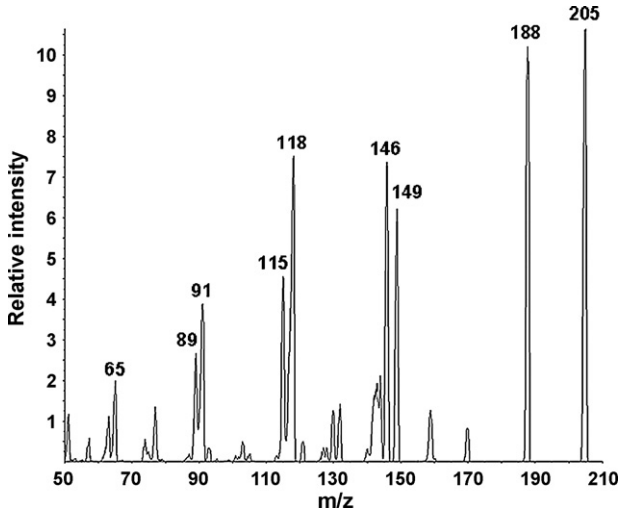

Fig. 1. MS/MS spectrum of tryptophan in the positive mode.

Japan). Interferon-γ (IFN-γ) was from PetroTech EC Ltd. (London, UK). Ultrapure water was prepared using a Milli-Q water system (Millipore-Waters, Milford, MA). HPLC-grade methanol was obtained from Nacalai Tesque (Kyoto, Japan). LN229 human glioma cells were obtained from American Type Cell Collection (Manassas, VA).

2.2. Instrumentation methodologies

LC/MS/MS was performed using a Shimadzu HPLC system (Kyoto, Japan) that was connected to a Sciex API 3000 tandem mass spectrometer (Concord, Ontario, Canada) outfitted with a turbo ion-spray ionization source operating in the positive mode. All compounds were separated over an Atlantis dC18 resin (150 mm × 2.1 mm i.d. column; 3 μm particle size) obtained from Millipore-Waters. The mobile phases consisted of 5 mM ammonium formate containing 0.01% trifluoroacetic acid (v/v) (A), and 100% methanol (B). The compounds were separated using a linear gradient that began at A/B = 100/0 and ended at 10 min with A/B = 30/70. The mobile phase was then held at A/B = 30/70 for 5 min. Finally, the mobile phase was returned to A/B = 100/0 at 15.1 min and the resin re-equilibrated with A/B = 100/0 for 5 min. The flow rate, at all times, was 0.15 mL/min and the injection volume was 10 μL.

To optimize the multiple reaction monitoring (MRM) sensitivity, 10 μM of each compound was infused into a mixture of 5 mM ammonium formate and methanol (50:50, v/v) at a rate of 10 μL/min with a Harvard syringe pump 11 (Harvard Apparatus, South Natick, MA). Nebulizing gas, curtain gas and collision gas flows were set at the instrument settings of 10, 6 and 6, respectively. The ion spray voltage was 5500 V and the temperature was 450 °C. The *m*/*z* range monitored was between 205 (precursor ion) and 146 (product ion) for tryptophan, 209 and 146 for kynurenine, 225 and 110 for 3-hydroxykynurenine, 154 and 80 for 3-hydroxyanthranilic acid, or 219 and 160 for tryptophan methyl ester. The MRM mode dwell time was 200 ms. The optimum voltage settings for the declustering potential (DP), focusing potential (FP), collision energy (CE) and collision exit potential (CXP) were 36, 200, 25 and 10 for tryptophan; 26, 150, 29 and 10 for kynurenine; 26, 160, 25 and 6 for 3-hydroxykynurenine; 21, 140, 37 and 14 for 3-hydroxyanthranilic acid; 26, 170, 27 and 10 for tryptophan methyl ester, respectively. Standard calibration curves were generated using 1/*X*-weighted linear regressions and then used to determine the concentrations of the unknown samples. Analyst version 1.4 software (Applied Biosystems, Foster City, CA) was used for data acquisition and processing.

Table 1
Extraction efficiencies and associated coefficients of variation for tryptophan metabolites from the culture medium

Compound	Added (μM)	CV (%)	Extraction efficiency (%)
Tryptophan	100	5.2	106
Kynurenine	10	3.5	111
3-Hydroxykynurenine	10	5.7	106
3-Hydroxyanthranilic acid	5	1.6	96
Tryptophan methyl ester	5	0.9	105

MEM containing 5% FBS was spiked with the indicated concentration of the listed compounds. After 10-fold dilution of the extract with 0.5N perchloric acid, the amount of each compound was determined using LC/MS/MS. The CVs ($n = 5$) and the extraction efficiencies (%) were then calculated.

2.3. Accuracy and precision of the assay

To determine the error limits, LN229 cells or culture media were spiked with tryptophan, 3-hydroxyanthranilic acid, kynurenine, 3-hydroxykynurenine, and tryptophan methyl ester at the indicated concentrations shown in Tables 1 and 2. Sample recovery is expressed as [((found concentration − endogenous concentration)/spiked concentration) × 100%] and the precision calculated as the coefficient of variation (CV, %).

2.4. Preparation of calibration standards

Stock solutions of tryptophan, kynurenine, 3-hydroxykynurenine, and tryptophan methyl ester were each prepared in ultrapure water. That of 3-hydroxyanthranilic acid was prepared in methanol. These solutions (1 mM) were stored at −80 °C for up to 2 months. Calibration standards (final concentrations: 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 μM of tryptophan; 0.02, 0.1, 0.2, 0.4, 1, 2, 4, 10 μM of kynurenine and 3-hydroxykynurenine; 0.1, 0.5, 1, 2, 5, 10 μM of 3-hydroxyanthranilic acid) were prepared daily from the stock solutions by serial dilutions with 0.5N perchloric acid. The least concentrated calibration standard with a signal-to-noise ratio of 10 defined the lower quantification limit that could be reliably measured.

2.5. Sample preparation from culture medium and cells

LN229 cells (2×10^6), grown in MEM supplemented with 5% FBS, penicillin (100 units/mL) and streptomycin (50 μg/mL), were stimulated with 100 units/mL IFN-γ. The culture medium or cell samples were acid-precipitated with 0.5N perchloric acid containing 0.5 μM tryptophan methyl ester for 10 min on ice. The samples were then centrifuged (15,000 × *g*, 10 min) and the supernatants stored at −80 °C. An equal volume of 1 M ammonium formate was added to each supernatant just before LC/MS/MS analysis.

Table 2
Extraction efficiencies and associated coefficients of variation for tryptophan metabolites from LN229 cells

Compound	Added (μM)	CV (%)	Extraction efficiency (%)
Tryptophan	100	1.2	92
Kynurenine	10	3.3	93
3-Hydroxykynurenine	10	3.8	83
3-Hydroxyanthranilic acid	5	1.8	93
Tryptophan methyl ester	5	6.7	90

Cells (2×10^6 cells) were spiked with 200 μL of the indicated concentrations of the listed compounds. After 10-fold dilution of the extract with 0.5N perchloric acid, the amount of each compound was determined using LC/MS/MS. The CVs ($n = 5$) and the extraction efficiencies (%) were then calculated.

Table 3
Concentrations of tryptophan metabolites from the culture medium and cells after IFN-γ treatment

	Culture medium (μM)		Cell (μM)	
Time after stimulation (h)	0	48	0	48
Tryptophan	48.5 ± 4.9	N.D.	33.3 ± 10.4	14.4 ± 2.4
Kynurenine	0.9 ± 0.0	32.2 ± 0.3	N.D.	344.5 ± 36.0
3-Hydroxykynurenine	N.D.	0.1 ± 0.0	N.D.	1.6 ± 0.2
3-Hydroxyanthranilic acid	N.D.	0.8 ± 0.1	N.D.	1.3 ± 0.1

Cells were stimulated with 100 units/mL of IFN-γ. The concentrations of tryptophan metabolites from the culture medium and cells were determined by LC/MS/MS as described in Section 2. The cellular concentrations of the metabolites were estimated from the packed volumes of the cells. Data are the mean ± S.D. ($n = 3$). N.D.: not detected.

3. Results and discussion

We recorded the mass spectra of 3-hydroxykynurenine, 3-hydroxyanthranilic acid, tryptophan, kynurenine and tryptophan methyl ester (an internal standard) in the positive mode because these compounds are barely detectable in negative mode. The precursor peaks for tryptophan, its metabolites and tryptophan methyl ester are the corresponding protonated molecular ions $[M+H]^+$. The major collision-induced fragments were identified for all compounds and their spectral sensitivities individually optimized. The tryptophan MS/MS spectrum is shown in Fig. 1. Using the optimized conditions, the compounds were first separated over the reverse-phase dC18 resin in a mobile phase consisting of a trifluoroacetic acid/ammonium formate–methanol gradient. Then, the isolated compounds were ionized using an electrospray source in preparation for mass spectroscopy and detected in the MRM mode. Chromatograms of the compounds are shown in Fig. 2A. Retention times for tryptophan, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and tryptophan methyl ester were 11.94, 9.74, 7.99, 12.36 and 13.77 min, respectively. Addition of 0.01% trifluoroacetic acid (v/v) to the 5 mM ammonium formate mobile phase decreased the lower limit of detection for 3-hydroxyanthranilic acid by 70%, but had significantly lesser effects on the lower limits of detection for the other compounds. By contrast, formic acid up to 1% concentration was found to slightly increase the lower limit of detection of the four compounds (data not shown).

We next determined the limits of quantification for the compounds. Data points of the X and Y axes represent the ratios of the concentrations of a compound to the concentration of the internal standard, and the ratios of the areas of a compound's product ion peak to the area of the product ion peak for the internal standard corresponding to 5 pmol, respectively. All compounds described in Section 2 displayed excellent linearity in calibration. The regression equations for these curves and their calculated correlation coefficients (given in parentheses) were as follows: tryptophan, $Y = 0.00983X + 0.00244$ (0.9921); kynurenine, $Y = 0.00758X + 0.00158$ (0.9919); 3-hydroxykynurenine, $Y = 0.00753X + 0.000723$ (0.9913); 3-hydroxyanthranilic acid, $Y = 0.0146X - 0.00278$ (0.9953). The lower limits of quantification were 0.01 μM for tryptophan, 0.02 μM for kynurenine and 3-hydroxykynurenine and 0.1 μM for 3-hydroxyanthranilic acid. Tryptophan, kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid were linear up to 10 μM and the concentrations of the compounds were measured within these ranges by diluting the samples. The lower limits of quantification of tryptophan, kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid by LC–ESI/MS/MS [14,15], electrochemical potential [16] and fluorescence methods [17] were 0.01 μM. Thus, except for 3-hydroxyanthranilic acid, sensitivity of detection of the other three compounds was similar using our method and the previous LC–ESI/MS/MS and electrochemical methods. Although the sensitivity of 3-hydroxyanthranilic acid is low, it can be measured in biological samples as shown in Table 3.

MEM medium or LN229 glioma cells were spiked with a known amount of each compound and then the compounds were quantified using our method. The results of these experiments were used to determine the accuracy and precision associated with each compound for our method. This data is summarized in Tables 1 and 2. The CVs are less than 7% and the recoveries range from 83 to 111%. The compounds were not degraded in the presence of perchloric acid for at least 7 days (data not shown). These results indicate that this method is accurate and precise.

Using this method, we examined whether we could simultaneously measure the kynurenine pathway metabolites in both the culture medium and cell lysates using glioma cells stimulated with interferon-γ (IFN-γ), an inducer of indoleamine 2,3-dioxygenase (IDO) that catalyzes the first and rate-limiting step of the kynurenine pathway. Chromatograms of culture medium stimulated with IFN-γ for 48 h are shown in Fig. 2B. IFN-γ treatment almost completely depleted the culture medium of tryptophan and resulted in an accumulation of tryptophan metabolites. Kynurenine was the major tryptophan metabolite to accumulate with minor amounts of 3-hydroxykynurenine and 3-hydroxyanthranilic acid (Table 3). Previously, these compounds have been measured separately [9–11,14–17]. This is the first report to simultaneously measure the compounds in a single analysis. As anticipated, the total amount of kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid did not exceed the initial amount of tryptophan in the culture medium. Similar results were obtained for the cell lysates. Tryptophan could still be detected in the cells even after treatment with IFN-γ. The intracellular concentrations of kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid were higher than those found in the medium (Table 3). The effects of IFN-γ treatment on kynurenine pathway metabolism have been investigated using various types of cells [18–22]. In most cases, IFN-γ induced a decrease in the level of tryptophan and a corresponding increase in the level of kynurenine. However, 3-hydroxykynurenine and 3-hydroxyanthranilic acid have been detected as minor products in macrophages [18–22]. Thus, our results were similar to those obtained for macrophages. The response to IFN-γ may differ depending on the cell type used.

In addition to tryptophan depletion, kynurenine pathway metabolites, such as 3-hydroxykynurenine and 3-hydroxyanthranilic acid, assume active roles in inhibiting proliferation and inducing apoptosis. It has been reported that an increased level of 3-hydroxykynurenine is observed in several neurodegenerative disorders such as Huntington's disease [23], Parkinson's disease [24], and acquired immunodeficiency syndrome dementia [25]. Kynurenine pathway metabolites may play important roles during inflammatory processes such as immure response and neurodegenerative disorders.

In this study, we describe an expanded method to simultaneously measure tryptophan and its metabolites and have successfully determined both the intra- and extracellular metabolites of the kynurenine pathway of IFN-γ stimulated glioma cells. This is the first report of a method to determine tryptophan and kynurenine pathway metabolites both intra- and extracellularly.

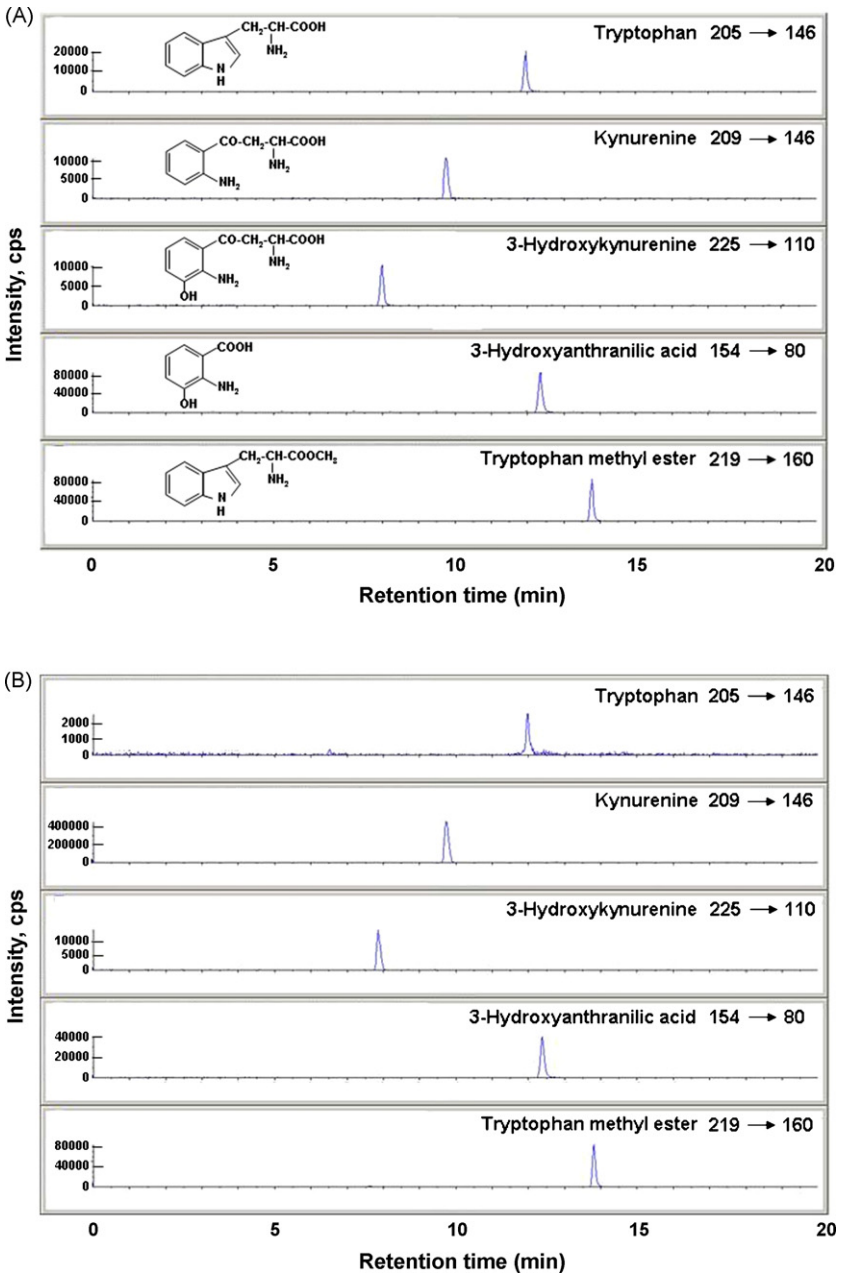

Fig. 2. Chromatograms of tryptophan and related compounds using the multiple reaction monitoring mode. (A) Standard: the amounts injected were 1 pmol for tryptophan, 2 pmol for kynurenine and 3-hydroxykynurenine, 10 pmol for 3-hydroxyanthranilic acid and 5 pmol for tryptophan methyl ester, respectively. Chemical structures of the compounds are shown in the inset. (B) The culture medium treated with IFN-γ for 48 h; the concentrations of tryptophan, kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid were 0, 32.2, 0.1 and 0.8 μM, respectively. The ion transitions for each compound are shown on the right hand side of each chromatogram.

Our results demonstrate a higher concentration of three metabolites of tryptophan in glioma cells after stimulation with IFN-γ. We believe our methodology could be applied to the simultaneous measurement of these compounds in serum or cerebrospinal fluid together with tissue samples. Such an approach should contribute to the efficient and accurate determination of *in vivo* data in order to elucidate the role of the kynurenine pathway in inflammatory diseases.

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