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(71) Applicant: **ST. JUDE CHILDRENS RESEARCH HOSPITAL** [US/US]; 262 Danny Thomas Place, Memphis, TN 38105 (US).

(72) Inventors: **PANI, Amar, K.**; 9341 Lake Bridge Drive, Lakeland, TN 38002 (US). **SMEYNE, Richard, Jay**; 10394 Hulsey Circle, Collierville, TN 38017 (US).

(74) Agents: **LICATA, Jane, Massey** et al.; Licata & Tyrrell P.C., 66 E. Main Street, Marlton, NJ 08053 (US).

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(54) Title: METHOD FOR DETECTION OF ADENOSINE AND METABOLITES THEREOF

(57) Abstract: This invention relates is a label-free, enzyme-free, aptamer-free method for simultaneously measuring adenosine, and its intracellular metabolites, e.g., AMP, ADP and ATP, using high pressure liquid chromatography coupled to electrochemical detector (HPLC-ECD).

Method for Detection of Adenosine and Metabolites Thereof

Introduction

[0001] This application claims the benefit of priority from U.S. Patent Application Serial No. 61/770,418, filed February 28, 2013, the content of which is incorporated herein by reference in its entirety.

Background

[0002] Purine and pyrimidine nucleosides and bases, the essential building blocks of nucleic acids, occur widely throughout the animal kingdom and underlie a number of critical functions including energy transduction, metabolism and cell signaling. One endogenous purine nucleoside, adenosine (ADO), plays an important role in a number of biochemical processes including energy transfer.

[0003] In the nervous system, ADO acts as a non-classical inhibitory neurotransmitter (Pedata, et al. (1983) *Neuropharmacol.* 22:609-614; Jackisch, et al. (1984) *Naunyn Schmiedebergs Arch. Pharmacol.* 327:319-25) and neuromodulator (Phillis & Wu (1981) *Prog. Neurobiol.* 16:187-239; Snyder (1985) *Annu. Rev. Neurosci.* 8:103-124). Alterations in adenosine or its signaling have been linked to a number of neurological disorders including epilepsy (Boison (2012) *Glia* 60:1234-43), Parkinson's disease (Wardas (2002) *Pol. J. Pharmacol.* 54:313-26; Schwarzschild, et al. (2006) *Trends Neurosci.* 29:647-54), schizophrenia (Boison, et al. (2012) *Neuropharmacology* 62:1527-43), panic disorder and anxiety (Hohoff, et al. (2010) *J. Psychiatr. Res.* 44:930-7), as well as drug abuse (Ferre, et al. (2007) *Prog. Neurobiol.* 83:332-47). Alterations in ADO have also been linked to changes in a sleep and arousal (Porkka-Heiskanen & Kalinchuk (2011) *Sleep Med. Rev.* 15:123-35) as

well as cognition and memory (Wei, et al. (2011) *Learn. Mem.* 18:459-74; Fredholm, et al. (2005) *Int. Rev. Neurobiol.* 63:191-270).

[0004] With the brain, extracellular ADO concentrations have been reported to be in the 30-400 nM range (Fredholm, et al. (1999) *Pharmacol. Rev.* 51:83-133; Zetterström, et al. (1982) *Neurosci. Lett.* 29:111-115). However, in response to cellular damage (*e.g.*, seizure or ischemia), these concentrations can quickly elevate (Zetterström, et al. (1982) *supra*; Berman, et al. (2000) *Brain Res.* 872:44-53), in some cases 7.5-31-fold (During & Spencer (1992) *Ann. Neurol.* 32:618-24), suggesting that ADO, in addition to signaling, also can have a neuroprotective function.

[0005] Adenosine functions by binding to and signaling through four known receptor subtypes (A1, A2A, A2B, and A3)(Gomes, et al. (2011) *Biochim. Biophys. Acta* 1808:1380-99; Sebastiao & Ribeiro (2009) *Handb. Exp. Pharmacol.* 471-534). One of the best-known compounds that acts via ADO signaling, and in particular by bind to the A2A receptor, is caffeine. This drug's stimulatory effects are primarily (although not entirely) credited to its inhibition of ADO via competitive inhibition of these receptors (Lazarus, et al. (2011) *J. Neurosci.* 31:10067-75), effectively blocking adenosine signaling. The subsequent reduction in ADO signaling leads to increased activity of other neurotransmitters including acetylcholine (Jin & Fredholm (1997) *Naunyn Schmiedebergs Arch. Pharmacol.* 355:48-56), noradrenaline (Allgaier, et al. (1991) *Naunyn Schmiedebergs Arch. Pharmacol.* 344:187-92), GABA (Kirk & Richardson (1995) *J. Neurochem.* 64:2801-9), dopamine (Ferre, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:7238-41) and glutamate (Rodrigues, et al. (2005) *J. Neurochem.* 92:433-41).

[0006] Conventionally, the ability to directly measure adenosine and its metabolites has been difficult, and has generally been carried out by proxy. For example, aptamer-based approaches have been described (Zhou & Zhao (2012) *Analyst* 137:4262-6; Liu & Yan (2012) *Biosens. Bioelectron.* 36:135-41; Li, et al. (2012) *Anal. Chem.* 84:2837-2842; Huang & Liu (2010) *Anal. Chem.* 82:4020-26; Yan, et al. (2009) *Talanta* 79:383-87; Kim, et al. (2009) *Chem. Commun.* 31:4747-9), as have label-based (Huang, et al. (2011) *Biosens. Bioelectron.* 29:178-83) and enzyme-based, *e.g.*, luciferase (Burgos, et al. (2012) *Anal. Chem.* 84:3593-8; Simpson, et al. (2008) *Lett. Appl. Microbiol.* 11:208-10)or S-adenosylhomocysteine hydrolase (WO 1999/034210; US 6,395,256) approaches.

[0007] More direct approaches have also been suggested. For example, a combined high pressure liquid chromatography (HPLC) separation and electrochemical detection method has been described for identifying adenosine and guanosine (Henderson & Griffin (1984) *J. Chromatgraph. A* 298:231-42). However, clean-up procedures were suggested to remove interfering material in biological samples. Similarly, a combined reversed-phase HPLC, gradient elution and electrochemical and UV detection method has been described for detecting adenosine, guanosine, inosine, guanine, hypoxanthine, xanthine and urate in mouse brain and serum, as well as in post-mortem human brain (Burdett, et al. (2012) *Biomed. Chromatograph.* Doi:10.1002/bmc.2760). Moreover, simultaneous detection of dopamine and adenosine has been described using a boron-doped diamond working electrode in combination with HPLC (Birbeck & Mathews (2012) *Pittcon* 2012, Abstract 1370-3; Birbeck & Mathews (2013) *Anal. Chem.* 85:7898-404).

Summary of the Invention

[0008] The present invention is a method for simultaneously detecting adenosine, and its metabolites, by (i) separating the components of a biological sample by reversed phase high pressure liquid chromatography under isocratic elution conditions; (ii) collecting fractions; and (iii) simultaneously detecting the levels of adenosine, and metabolites thereof, in each fraction with a coulometric electrochemical detector. In one embodiment, the method also includes the step of comparing the detected levels of adenosine, and metabolites thereof, with a standard curve to quantitate the amount of adenosine, and metabolites thereof, present in the biological sample. In other embodiment, step (i) is carried out under acidic conditions or with a mobile phase containing sodium perchlorate and acetonitrile at a pH in the range of 2 to 4. In a further embodiment, the adenosine metabolites include adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate.

Detailed Description of the Invention

[0009] Alterations in the levels of adenosine have been correlated with a number of neurological diseases including Parkinson's disease, Schizophrenia, Epilepsy, Stroke, Panic Disorder and other Psychiatric diseases as well as immunological syndromes. Measurement of adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP) and adenosine (ADO) can be used to examine cellular bioenergetics. Therefore, a rapid means for detecting adenosine and its metabolites provides a means for diagnosing such diseases as well as for monitoring therapeutic treatment of the same.

[0010] A label-free, enzyme-free, aptamer-free method for simultaneously measuring the purine neurotransmitter adenosine, and its intracellular metabolites, *e.g.*, AMP, ADP and ATP, using high pressure liquid chromatography coupled to electrochemical detector (HPLC-ECD) has now been developed. Using this method, adenosine and its nucleoside metabolites were measured from several brain regions, including brainstem, cerebellum, cortex, hippocampus, olfactory bulb, substantia nigra and striatum, in a number of common inbred mouse strains (C57BL/6, Swiss-Webster, FVB/J, 129P/J, and BALB/c) that are typically used to develop neurodegenerative disease models. It was found that levels of adenosine and the nucleoside metabolites vary significantly among the different regions and mouse strains examined. In regard to adenosine, C57BL/6 mice had the lowest levels in brain while BALB/c mice have the highest. It is possible that differences in baseline adenosine levels contribute to differential strain effects seen in the expressed phenotypes of neurodegenerative mouse models.

[0011] According to one embodiment of the invention, the method comprises the steps of: separating the components of a biological sample by reversed phase HPLC under isocratic elution conditions, collecting fractions, and simultaneously detecting the levels of adenosine, and intracellular metabolites thereof, in each fraction with a coulometric electrochemical detector. In some embodiments, that method also includes comparing the detected levels with a standard curve to determine the amount of total adenosine, and intracellular metabolites thereof, present in the original sample. In another embodiment, the method consists of the steps of: separating the components of a biological sample by reversed phase HPLC under isocratic elution conditions; collecting fractions; simultaneously

detecting the levels of adenosine, and intracellular metabolites thereof in each fraction with an coulometric electrochemical detector; and comparing the detected levels with a standard curve to determine the amount of total adenosine, and intracellular metabolites thereof, present in the original sample. The method of the invention is "label-free," "enzyme-free," and "aptamer-free" in the sense that the method does not include the use of a label, enzyme or aptamer to detect adenosine or its metabolites.

[0012] As used herein, a biological sample is intended to mean any biological fluid, or fraction thereof; or an extract of a cell or tissue, or a supernatant or fraction thereof, that contains adenosine, or one or more intracellular metabolites thereof. A sample can be, for example, obtained from an individual or can be derived from such a specimen. For example, a sample can be a supernatant obtained from a minced tissue section obtained by biopsy. Exemplary samples can also include extracts of cultured fibroblasts and cultured amnionic fluid cells. A sample can also be a biological fluid specimen such as urine, blood, plasma, serum, saliva, semen, sputum, cerebral spinal fluid, tears, mucus, and the like. A sample can be further fractionated, if desired, to a fraction containing particular cell types. For example, a blood sample can be fractionated into serum or into fractions containing particular types of blood cells such as red blood cells or white blood cells (leukocytes). If desired, a sample can be a supernatant from a combination of samples from an individual such as a combination of a tissue and fluid sample, and the like. In particular embodiments, the biological sample has been treated with an acid (*e.g.*, perchloric acid or sulfosalicylic acid) to deproteinate the sample.

[0013] In reversed phase HPLC, a solute molecule binds to an immobilized hydrophobic molecule in a polar solvent. This partitioning occurs as a result of the solute molecule tending to have hydrophobic patches at its surface, and binding via those patches to the matrix. A mobile phase dissociates the bound molecule at a point at which the hydrophobic interaction between the exposed patches and the immobilized matrix is less favorable than the interaction between the bound molecule and the solvent. The molecule releases from the matrix and elutes. In certain embodiments of this invention, reversed phase HPLC separation of the components of the biological sample is carried out with a DHBA column (Thermo-Fisher Scientific).

[0014] In accordance with this invention, elution of the components of the biological sample is carried out isocratically (*i.e.*, the mobile phase composition does not change during the separation process). As is conventional in the art, the mobile phase of the invention is composed of an aqueous component (*e.g.*, water or buffer) and a solvent. Desirably, the solvent is an organic solvent such as methanol, tetrahydrofuran or acetonitrile and is present at a level of between 2 and 20%, or more preferably in the range of 2 to 10% (v/v). In certain embodiments, the mobile phase of the invention contains sodium perchlorate and acetonitrile. The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Therefore, in certain embodiments of this invention, the pH of the mobile phase is acidic, *e.g.*, in the range of 2-5 or 2-4. In particular embodiments, the pH of the mobile phase is in the range of 2 to 3, which was found to provide the best separation and resolution of peak amplitudes. In other

embodiments, separation and elution are carried out at room temperature.

[0015] Once the components of the biological sample elute from the reversed phase column, fractions are collected. Collection can be manual or automated and is typically carried out at room temperature. Once collected, the fractions are analyzed by an electrochemical detector. Electrochemical detectors respond to substances that are either oxidizable or reducible and the electrical output results from an electron flow caused by the chemical reaction that takes place at the surface of the electrodes. The detector normally has three electrodes, the working electrode (where the oxidation or reduction takes place), the auxiliary electrode and the reference electrode (which compensates for any change in the electrical conductivity of the mobile phase). There are two modes of operation coulometric detection and ampiometric detection. If the reaction at the electrode surface exhausts all the reactant and the current becomes zero, the total charge that passes will be proportional to the mass of solute detected. In this respect, this process is called coulometric detection. If the mobile phase is flowing past the electrodes, the solute will be continuously replaced as the peak passes through the detector. While there is solute present between the electrodes, a current will be maintained (albeit varying in magnitude). The process is called ampiometric detection. In particular embodiments of this invention, the fractions are subjected to coulometric detection.

[0016] While the electrochemical detector read-out can directly be used to determine the presence, absence or relative level of adenosine, or a metabolite thereof, in a biological sample, in some embodiments, the levels detected with the electrochemical detector are compared with a

standard curve to quantitate the amount of total adenosine, and intracellular metabolites thereof, present in the original sample. The standard curve can be generated prior to or after the biological sample is separated and can be composed of one component, *e.g.*, adenosine, or a combination of components, *e.g.*, ADO, ATP, ADP and/or AMP. Alternatively, an internal standard can be added to the biological sample (*e.g.*, 3,4-dihydroxybenzylamine).

[0017] Using the method of this invention, the levels of adenosine, and intracellular metabolites thereof, are simultaneously detected. In particular, the levels of one or more of adenosine, AMP, ADP or ATP. In accordance with this invention, it is possible to achieve sensitivities in the range of 1-1000 μmol/liter of adenosine, AMP, ADP and/or ATP. Given this level of detection, the claimed method finds application in the diagnosis of diseases or conditions resulting from, or associated with, alterations in the levels of adenosine. Such diseases or conditions include, but are not limited to, Parkinson's disease, Schizophrenia, Epilepsy, Stroke, Panic Disorder and other Psychiatric diseases as well as immunological syndromes.

[0018] The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Neurochemical Analysis of Adenosine in Brain

[0019] A high throughput neurochemical assay has now been developed for the detection and quantification of adenosine (a brain neurotransmitter and modulator involved in various neurological diseases) by high performance liquid chromatography and an electrochemical detector (HPLC-ECD).

[0020] *Chemicals*. Optima LC/MS grade Acetonitrile, Methanol, Perchloric acid and Phosphoric acid were

purchased from ThermoFisher Chemicals (NJ). Adenosine (ADO), adenosine 5'-monphosphate (ADP), adenosine 5'-monophosphate (AMP), sodium dihydrogen phosphate, potassium phosphate, sodium acetate and sodium perchlorate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Adenosine triphosphate disodium (ATP) was purchased from LKT laboratories, St. Paul, MN).

[0021] *Animals*. Male C57BL/6J, FVB/NJ, 129P/J and BALB/c were purchased from the Jackson Labs (Bar Harbor, ME), while Swiss Webster (SW) mice were purchased from Harlan (Indianapolis, IN). These mice were housed five per cage in the AALAC-certified vivarium at St Jude Children's Research Hospital and were maintained on a 12 hour light-dark cycle (6:00 AM-6:00 PM) in a temperature- and humidity-controlled room with food and water ad libitum. All of the mice in this study were 6-12 months old and weighed between 23-29 grams at the time of sacrifice.

[0022] *Preparation of Tissue for HPLC-ECD Analysis of Adenosine*. Mice were deeply anesthesized with AVERTIN (tribromoethanol) until all deep tendon and corneal reflexes were absent. Mice were subsequently transcardially perfused with ice-cold saline to remove circulating blood, after which brains were rapidly dissected from the calvaria. Brains were then placed in a cooled brain matrix (Model BS-AL-5000C, Braintree Scientific, Braintree, MA) and sliced into 2 mm thick sections. Six brain regions were subsequently subdissected using the following coordinates, olfactory bulb, cerebral cortex (Bregma: -1.00- -3.00mm), striatum (Bregma: +0.00-+2.00mm), hippocampus (Bregma: -1.00- -3.00mm), substantia nigra (Bregma: -2.00- -4.00) and brainstem (Bregma: -5.00- -7.00). The whole process took approximately 7 minutes/mouse.

[0023] Once the tissues were dissected, the samples were immediately frozen on dry ice, placed into pre-weighed 1.5 ml tubes and stored at -80°C. On the day of analysis, tissue was weighed, thawed and minced in 200 microliters of mobile phase (see below). Tissue samples were homogenized by a hand-held homogenizer (Pellet Pestle Motor, ThermoFisher Scientific) and centrifuged at 13,700 rpm for 27 minutes at 4°C. The resultant pellet and supernate were separated, frozen and stored at -80°C for no more than two weeks prior to chromatographic analysis.

[0024] *Preparation of Standard Solutions and Biological Samples*. Separate stock solutions of ADO, ATP, ADP and AMP were prepared in Pani mobile phase at 1 mg/ml and stored at 4°C. The working standard solutions were prepared daily by diluting the stock solutions to 1.25, 2.5, 5.0 and 10.0 µg/ml solutions. Ten µl of each stand solution was injected using an autosampler to generate a simulatenous standard curve immediately prior to HPLC-ECD analysis of the brain samples.

[0025] The brain samples were prepared using diluents composed of Pani mobile phase (75%) with 0.3N perchloric acid (25%), which gave a recovery of approximately 90% compared to standards.

[0026] *Comparison of ADO Separation and Quantification Using Different Stationary and Mobile Phases*. Two of the key determinants of separation and quantification of purines in HPLC-ECD is the use of the stationary phase (chromatographic column) in combination with the proper electrolyte (mobile phase). Selection of the proper mobile phase is important since one has to properly dissolve the compounds to be measured, while the proper column function to differentially retain solutes based upon hydrophobic interactions with its stationary phase.

[0027] Two protocols describing HPLC-ECD identification of adenosine have been described (Henderson & Griffin (1984) J. Chromatograph. 298:231-242; Birbeck, et al. (2012) Pittcon 2012. Orlando. pp. 70). However, these references did not describe the direct and simultaneous detection of adenosine as well as its metabolites, ATP, ADP and AMP. In contrast, the instant HPLC-ECD protocol was developed for simultaneous detection of ADO, ATP, ADP and AMP. Four different columns and five different mobile phases were empirically tested. Each column and mobile phase was used in combination such that 20 possible pairs were tested.

[0028] The columns included: (1) ESA, HR80, 4.6 x 80 mm, C18, 3 micron particle size; (2) ESA, MD-150, 3.2 x 150 mm, C18, 3 micron particle size; (3) Kinetex 2.1 x 50 mm, C18, 2.6 micron particle size, and (4) ESA, DHBA-3.2 x 250mm, C18, 5 micron particle size.

[0029] The mobile phases tested were: (1) sodium phosphate monobasic & dibasic, 1-octanesulphonic acid, acetonitrile and methanol, pH 3.85; (2) sodium phosphate monobasic, 1-octanesulphonic acid; acetonitrile, pH 3.85, (3) potassium phosphate, 1-octanesulphonic acid, EDTA, acetonitrile and methanol, pH 3.5; (4) sodium phosphate, potassium phosphate, 1-octanesulphonic acid, EDTA, methanol and acetonitrile, pH 3.5; (5) a mobile phase (Pani mobile phase) composed of 19.0 mM sodium perchlorate (Fischer Scientific) and 4.0% (v/v) acetonitrile (Optima, LC/MS grade) prepared in double distilled, deionizer, autoclaved water tested at 4 different pH values (1.75, 2.75, 3.75 and 7.0), each adjusted to the target pH with phosphoric acid (85%, Fisher). Buffer (5) was filtered prior to use through a 0.22 μM filter membrane under vacuum and degassed through an ESA model DG4 degasser, and pumped at a rate of 0.5

ml/min, producing a background pressure of approximately 161 BARs.

[0030] Empirical data demonstrated good separation of ATP, ADP, AMP and ADO upon elution only with the ESA, DHBA-3.2 x 250mm, C18, 5 micron particle size column in combination with the newly formulated mobile phase (Pani mobile phase (5), pH 2.75).

[0031] Quantitative *Analysis of Adenosine, AMP, ADP and AMP*. Adenosine, AMP, ADP and AMP were analyzed using high performance liquid chromatography with electrochemical detection. Briefly, the chromatographic system included a solvent delivery system (ThermoFisher Scientific, model 584 pump) and an autosampler (ThermoFisher Scientific, model 542) equipped with an injection valve containing a 15 μl sample loop. ADO, ATP, ADP and AMP detection was accomplished by means of a coulometric electrochemical detector (Coulochem III, ThermoFisher Scientific). The analytical cell (Boron Doped Diamond (BDD), Electron North America, LLC, Model 5040) was used versus a hydrogen/palladium reference electrode and was set to +1300mV. This voltage was determined by empirical measurement of all four compounds and then balancing the ratio of oxidative potential to noise at each voltage. Chromatographic separations were performed on a DHBA column (diameter 250x3.2 mm, Thermo-Fisher Scientific) in-line with a pre-column column (Thermo-Fisher), and the entire system was run at ambient temperature.

[0032] The signal from the electrochemical detector was recorded using a model SS420x integration device (Scientific Software Inc.) and the retention time of ADO, ATP, ADP and AMP standards (Sigma) were empirically determined. After identification of retention time for each compound, concentration curves, ranging from 0 to 100 ng,

were generated and linearity of detection of the analytes was confirmed. Identification of ADO, ATP, ADP and AMP were further confirmed by spiking random samples with external standards and finding no peak shift. ADO, ATP, ADP and AMP concentrations in tissues were quantified by comparing the peak areas of the sample chromatograms with external standard chromatograms. Additionally, re-extraction of the pellets generated during the initial extraction was performed to determine if ADO, ATP, ADP and AMP had been fully extracted. HPLC-ECD analysis of the re-extracted supernatant found no ADO, ATP, ADP or AMP, and on this basis, it was concluded that the peaks co-eluting from mice brain samples, in comparison to the external standard chromatogram, represented a true picture of the levels of ADO and its metabolites in the mouse brain.

Example 2: Determination of ADO, ATP, ADP and AMP

[0033] To determine the retention time of ADO, ATP, ADP and AMP, purified compounds, *i.e.*, adenosine (99% pure, Sigma, St. Louis, MO), adenosine triphosphate disodium (ATP, LKT Laboratories, St. Paul, MN), adenosine 5'-diphosphate (ADP, Sigma), and adenosine 5'-monophosphate, (AMP, Sigma) were dissolved in the Pani mobile phase at four different pH values (1.75, 2.75, 3.75 and 7.0). Ten microliters of combined standards and/or samples at each pH were injected into the HPLC at +1300mV with a flow rate of 0.5 ml/min. It was observed that the best separation and resolution of the peak amplitude occurred at 2.75. Thus, the Pani mobile phase at pH 2.75 was used for all subsequent analyses. The retention time of ADO, ATP, ADP and AMP were subsequently empirically determined. To demonstrate that each of these could be simultaneously detected on a single chromatogram, ten microliters of a 10 mg/ml solution of an equal mixture

of ADO, ATP, ADP, and AMP was analyzed. Four distinct peaks showing the individual compounds were resolved and there was no significant difference in the HPLC retention times when compared to their individual elutions.

Example 3: Quantification of ADO, ATP, ADP and AMP in Brain

[0034] The levels of ADO, ATP, ADP and AMP (pg/mg wet weight of tissue) in six different brain regions of C57BL/6J mice and their contribution to the total purine are listed in Table 1.

TABLE 1

Brain Region	Adenosine	ATP	ADP	AMP	Total Pool
Olfactory Bulb	3041± 473 (1%)	29865± 1941 (5%)	16684± 2058 (3%)	618096± 48753 (96%	667686
Cerebral Cortex	12620± 2065 (7%)	89799± 14699 (52%)	12620± 4462 (7%)	56682± 4274 (34%)	171721
Striatum	184669± 25677 (7%)	682872± 65889 (24%)	289646± 15151 (11%)	1582489± 75113 (58%)	2739676
Hippocampus	150597± 29364 (5%)	1673536± 194710 (52%)	469903± 38866 (15%)	938762± 1392562 (29%)	3232798
Substantia Nigra	16029± 2265 (3%)	92867± 4860 (19%)	60929± 3930 (13%)	308429± 23553 (65%)	478254
Cerebellum	9111± 1877 (9%)	35650± 5205 (36%)	27518± 1816 (28%)	26887± 3009 (27%)	99166

[0035] Of the six regions examined, the striatum and hippocampus had the highest amounts of total ADO, with cerebral cortex and substantia nigra having intermediate levels, while the olfactory bulb and cerebellum had the lowest levels measured. A similar pattern was observed for ATP, ADP and AMP pools.

Example 4: Quantification of ADO in the CNS of Different Mouse Strains

[0036] The levels of ADO (pg/mg wet weight of tissue) in 7 different brain regions of five different strains of mice were determined. The statistical significance of the changes among the strains and regions was assessed by ANOVA followed by Bonferroni post hoc analysis using SIGMASTAT software.

In general, the levels of ADO in the brain of each of the strains examined at basal conditions were low, with the exception of the BALB/c strain that had relatively higher ADO levels. In addition to differing levels of ADO among strains, there was also variation in the region examined. Regionally, the highest levels of ADO were seen in the striatum and olfactory bulb and lowest levels were in the brainstem. Significant differences were found among mouse strains in each region examined.

[0037] In olfactory bulb, significant differences were seen between the 5 strains ($F=32.57$, $p \leq 0.0001$) with ADO in BALB/c significantly increased compared to the other 4 strains examined. The order of ADO from highest to lowest in the olfactory bulb was BALB/c -> SW -> FVB/NJ -> 129P/J -> C57BL/6J. The increased ADO in BALB/c ranged from 532% higher than that measured in SW to a 17,566% increase compared to C57BL6/J.

[0038] In the striatum, significant differences were noted between the 5 mouse strains ($F=26.67$, $p \leq 0.0001$), with the highest levels of ADO in SW and BALB/c and lowest levels in C57BL6/J. The order of ADO from highest to lowest in striatum was BALB/c -> SW -> FVB/NJ -> 129P/J -> C57BL/6J. The increased ADO in BALB/c ranged from 162% higher than that measured in BALB/c to a 2480% increase compared to C57BL/6J.

[0039] In the cerebral cortex, significant differences were noted between the 5 mouse strains ($F=9.322$, $p \leq 0.0001$) with the higher levels of ADO in SW and BALB/c and lower levels in 129P/J, C57BL/6J and FVB/NJ. The order of ADO from highest to lowest in the cerebral cortex was SW -> BALB/c -> 129P/J -> C57BL/6J -> FVB/NJ. The increased ADO in SW ranged from 255% higher than that measured in 129P/J to a 415% increase compared to FVB/NJ.

[0040] In hippocampus, significant differences were noted between the 5 mouse strains ($F=16.70$, $p \leq 0.0001$), with the highest levels of ADO in 129P/J and BALB/c and lowest levels in SW. The order of ADO from highest to lowest in hippocampus was BALB/c -> 129P/J -> C57BL/6J -> FVB/NJ -> SW. The increased ADO in BALB/c ranged from 567% higher than that measured in 129P/J to 2787% increase compared to SW.

[0041] In substantia nigra, significant differences were noted between the 5 mouse strains ($F=22.73$, $p \leq 0.0001$), with the highest levels of ADO in SW and BALB/c and lowest levels in C57BL/6J and 129P/J. The order of ADO from highest to lowest in substantia nigra was BALB/c -> SW -> FVB/NJ -> 129P/J -> C57BL/6J. The increased ADO in BALB/c ranged from 258% higher than that measured in FVB/NJ to a 701% increase compared to C57BL/6J.

[0042] In brainstem, significant differences were seen among the 5 groups ($F=39.81$, $p \leq 0.0001$), with the highest levels of ADO in SW and BALB/c and lowest levels in C57BL/6J. The order of ADO from highest to lowest in brainstem was BALB/c -> SW -> FVB/NJ -> 129P/J -> C57BL/6J. The increased ADO in BALB/c ranged from 951% higher than that measured in FVB/NJ to an 1139% increase compared to C57BL/6J.

[0043] In cerebellum, significant differences were seen among the 5 groups ($F=44.20$, $p \leq 0.0001$), with the highest levels of ADO in BALB/c and lowest levels in C57BL/6J. The order of ADO from highest to lowest in brainstem was BALB/c -> SW -> C57BL/6J -> SW -> FVB/NJ. The increased ADO in BALB/c ranged from 281% higher than that measured in 129P/J to a 793% increase compared to FVB/NJ.

What is claimed is:

1. A method for simultaneously detecting adenosine, and its metabolites in a biological sample, comprising

(i) separating the components of a biological sample by reversed phase high pressure liquid chromatography under isocratic elution conditions;

(ii) collecting fractions; and

(iii) simultaneously detecting the levels of adenosine, and metabolites thereof, in each fraction with a coulometric electrochemical detector.

2. The method of claim 1, further comprising the step of comparing the detected levels of adenosine, and metabolites thereof, with a standard curve to quantitate the amount of adenosine, and metabolites thereof, present in the biological sample.

3. The method of claim 1, wherein step (i) is carried out under acidic conditions.

4. The method of claim 1, wherein step (i) is carried out with a mobile phase containing sodium perchlorate and acetonitrile at a pH in the range of 2 to 4.

5. The method of claim 1, wherein the metabolites comprise adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate.

6. A method for simultaneously detecting adenosine, and its metabolites, in a biological sample, consisting of:

(i) separating the components of a biological sample by reversed phase high pressure liquid chromatography under isocratic elution conditions;

(ii) collecting fractions;

(iii) simultaneously detecting the levels of adenosine, and metabolites thereof, in each fraction with a coulometric electrochemical detector; and

(iv) comparing the detected levels of adenosine, and metabolites thereof, with a standard curve to quantitate the amount of adenosine, and metabolites thereof, present in the biological sample.

7. The method of claim 6, wherein step (i) is carried out under acidic conditions.

8. The method of claim 6, wherein step (i) is carried out with a mobile phase containing sodium perchlorate and acetonitrile at a pH in the range of 2 to 4.

9. The method of claim 6, wherein the metabolites comprise adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/19215

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/52; G01N 1/18; B01D 15/08 (2014.01)
USPC - 73/61.55, 863.21; 554/175
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61K 31/52; G01N 1/18, 1/34, 1/40, 30/14; B01D 15/08, 15/32; C07H 19/16 (2014.01)
USPC: 73/61.55, 863.21; 554/175

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Scholar; Google; ProQuest; Separate, isolate, detect, purine, adenosine, metabolites, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), electrochemical detection

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	(BETTO, P et al.) Simultaneous high-performance liquid chromatographic determination of adenosine and dopamine in rat striatal tissue with combined ultraviolet absorbance and electrochemical detection. Journal of Chromatography B. 1994. Vol. 662. pages 21-25; page 22, left column, lines 8-15; page 22, left column, 'chromatographic system'; page 22, right column, lines 10-13; page 2, right column, 'paragraph 2'; page 23, left column 'recovery and precision'; page 23, left column 'results and discussion'; abstract.	1-3, 6-7 ---- 4-5, 8-9
Y	(SHIBUE, M et al.) The perchlorate anion is more effective than the trifluoroacetate anion as an ion-pairing reagent for reversed-phase chromatography of peptides. Journal of Chromatography A. 2005. Vol. 1080. pages 49-57; page 2, lines 10-11; page 2, 'column and HPLC conditions'; abstract.	4, 8
Y	(KHLYNTSEVA, SV et al.) Methods for the determination of adenosine triphosphate and other adenine nucleotides. Journal of Analytical Chemistry. 2009. Vol. 64. pages. 657–673; page 657, left column, paragraph 3; page 659, right column, 'chromatographic methods'; page 662, left column, lines 1-4; page 662, left column, last paragraph	5, 9
A	(PANI, AK et al.) Neurochemical measurement of adenosine in discrete brain regions of five strains of inbred mice. PLoS ONE. March 2014. Vol. 9. e92422. entire document	1-9

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2014 (15.05.2014)	02 JUN 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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