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# HPLC analysis of para-aminosalicylic acid and its metabolite in plasma, cerebrospinal fluid and brain tissues

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ABSTRACT

Para-aminosalicylic acid (PAS), an approved drug for treatment of tuberculosis, is a promising therapeutic agent for treatment of manganese (Mn)-induced parkinsonian syndromes. Lack of a quantifying method, however, has hindered the clinical evaluation of its efficacy and there upon new drug development. This study was aimed at developing a simple and effective method to quantify PAS and its major metabolite, N-acetyl-para-aminosalicylic acid (AcPAS), in plasma, cerebrospinal fluid (CSF) and tissues. Biological samples underwent one-step protein precipitation. The supernatant was fractionated on a reversed-phase C18 column with a gradient mobile system, followed by on-line fluorescence detection. The lower limits of quantification for both PAS and AcPAS were 50 ng/ml of plasma and 17 ng/g of tissues. The intra-day and inter-day precision values did not exceed 5% and 8%, respectively, in all three matrices. The method was used to quantify PAS and AcPAS in rat plasma and brain following a single iv injection of PAS. Data showed a greater amount of PAS than AcPAS in plasma, while a greater amount of AcPAS than PAS was found in brain tissues. The method has been proven to be sensitive, reproducible, and practically useful for laboratory and clinical investigations of PAS in treatment of Mn Parkinsonism.

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## 1. Introduction

Occupational exposure to manganese (Mn) in mining, ceramics, welding and dry battery industries is known to induce neurodegenerative disorder, clinically called manganism, a syndrome similar, but not identical, to Parkinson's disease [1–3]. In addition to typical movement disorder, the Mn-exposed patients exhibit the distorted mental functions such as memory loss, apathy and even psychosis [1,4]. These devastating clinical syndromes usually last for a life time; thus developing an effective and viable therapeutic agent for treatment of Mn-induced neurotoxicity is in urgent need.

Chelating therapy is commonly used as a strategy to relieve the body burden of metal exposure; however, the therapeutic outcomes for Mn are disappointing. For example, calcium sodium ethylenediaminetetraacetate ($CaNa_2EDTA$) is a common choice for metal exposure in clinics [5]. When used for Mn treatment, it does not significantly improve clinical syndromes [1,6–8], because of its poor permeability to cross brain barriers [9]. PAS (4-amino-2-hydroxybenzoic acid) is a U.S.-FDA approved drug used as the second-line medicine for the treatment of multidrug-resistant tuberculosis [10]. Ky et al. first reported an effective treatment of chronic severe Mn poisoning by PAS [11]. Jiang et al. further reported a successful prognosis with PAS in a 17-year follow-up study [12]. Additional clinical studies mainly conducted among Chinese patients have come to the similar conclusion [13–15]. Despite of these successful clinical treatments, our knowledge on how PAS exerts its therapeutic effectiveness, to what extent PAS is metabolized in the body, and whether PAS or its metabolites are capable of passing across the blood–brain barrier, is surprisingly incomplete. This is due, in a large part, to the lack of effective methodology to quantify PAS and pertinent metabolites in body fluids as well as in tissues.

Several methods have been developed for determination of PAS in blood. Unverricht et al. applied paper chromatography to analyze PAS [16]. Pemberton et al. [17] and Lianidou and Ioannou [18] used spectrofluorimetric methods for direct estimation of PAS in urine. Cummins et al. quantified N-acetyl-para-aminosalicylic acid (AcPAS), a major metabolite of PAS, in human urine by capillary zone electrophoresis method [19]. Gennaro et al. and later Vasbinder et al. developed a reversed-phase high-performance liquid

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chromatography (RP-HPLC) method [20] and ion-pair HPLC [21], respectively, to determine PAS. Noticeably, however, most, if not all, of the methods in literature were established to determine PAS in plasma for the purpose of its pharmacokinetic study [22–24]. To the best of our knowledge, quantitative assays for screening the permeability of PAS and its metabolites across brain barriers in brain tissues are still not available. Since the efficacy of PAS therapy may rely on the ability of parent PAS and/or its metabolites that enter brain parenchyma and distributed in different brain regions, determining PAS and its metabolites in blood and brain tissue has become critical for clinical evaluation of PAS safety in animal models and human subjects, as well as for the mechanistic investigation of PAS action in order to develop more effective new drug molecule(s) for manganism therapy.

The purpose of this study was to develop a reliable, sensitive, and rapid method for simultaneous determination of PAS and its major metabolite AcPAS in rat blood and brain tissues, and to use this method to investigate brain regional distribution of PAS and AcPAS following intravenous administration of PAS. Development of such a method should not only be highly desirable for quantification of the cerebral pharmacokinetics of PAS in animal models, but also offer a practical method for clinical monitoring of PAS in Mn-exposed patients.

## 2. Materials and method

### 2.1. Materials

All chemicals including para-aminosalicylic acid (4-amino-2-hydroxybenzoic acid, PAS), 5-aminosalicylic acid (5-amino-2-hydroxybenzoic acid), 2-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), polyacrylamide, and tetramethyl-ethylenediamine (TEMED) were purchased from Sigma Chemicals (St. Louis, MO). All reagents were of analytical grade, HPLC grade or the best available pharmaceutical grade. HPLC-grade water was prepared using NANOpure DIamond Ultrapure Water Systems (Barnstead International, Dubuque, IA). N-acetyl-para-aminosalicylic acid (AcPAS), a major PAS metabolite, was synthesized in our laboratory as described below.

N-acetylated derivative, AcPAS was synthesized from the parent PAS using acetic anhydride as acetylating agent, following a previously described procedure developed by Cummins et al. [19]. Briefly, PAS was dissolved in hydrochloric acid and stirred with acetic anhydride overnight. A 3 g precipitate was filtered, washed with distilled water (50 ml), air-dried, and then dissolved in the minimum amount of 0.1 M sodium hydroxide. The solution was stirred overnight at room temperature, after which the concentrated hydrochloric acid was used to adjust the pH of the solution to 2.0. The solution was then extracted with ethyl acetate ($2 \times 100$ ml) to get rid of potential O-acetylated derivative and the extracts were dried over anhydrous sodium sulphate, filtered, and concentrated to dryness under reduced pressure on a rotary evaporator. Hexane was then used to wash the solid residue from the flask and filtered; the procedure yielded 1.268 g N-AcPAS. The identity of individual products was confirmed by HPLC–MS, NMR and HPLC-DAD. The purity of N-acetylated derivative was found to be more than 99.1%.

### 2.2. HPLC-fluorescence (HPLC-FL) analysis

A Waters 2695 XE separations module liquid chromatographic system equipped with a built-in autosampler for 120 samples and a Waters 2475 multi λ fluorescence detector was used for HPLC analysis. Separation was accomplished using a reversed-phase Econosphere C18 column (5 μm, $250 \times 4.6$ mm) protected by a Spherisorb guard column (5 μm, $10 \times 4.6$ mm). Both analytical and guard columns were purchased from Alltech (Deerfield, IL).

**Table 1**
Gradient program for HPLC separation of PAS and AcPAS.

| Time (min) | Mobile phase | |
|---|---|---|
| | *A* (%) | *B* (%) |
| 0 | 90 | 10 |
| 2 | 90 | 10 |
| 5 | 85 | 15 |
| 15 | 80 | 20 |
| 18 | 20 | 80 |
| 19.5 | 90 | 10 |
| 25 | 90 | 10 |

Solvent A: equal molar concentration of both monobasic and dibasic potassium salts with a pH of 3.5 adjusted by phosphoric acid. Solvent B: methanol.

The optimized method used a binary gradient mobile phase with 17.5 mM potassium phosphate buffer as the solvent A (equal molar concentration of both monobasic and dibasic potassium salts with a pH of 3.5 adjusted by phosphoric acid) and methanol as the solvent B. The time program of the gradient is described in Table 1. Samples were injected and the separation was performed at room temperature at a flow rate of 1.0 ml/min with pressure between 2200 and 2500 psi. A standard sample of PAS was scanned for fluorescent spectra by a Spectra Max M2$^e$ (Molecular Devices, Sunnyvale, CA) and typical spectra for both excitation and emission scans are presented in Fig. 1. From the scanning data, the HPLC detector was set at an excitation wavelength of 337 nm and an emission wavelength of 432 nm. Empower Version Build 1154 (Waters Corporation, Milford, MA) was used for system control, data acquisition and analysis.

### 2.3. Preparation of calibration standards and quality control (QC) samples

Stock solutions of PAS and AcPAS were prepared separately by dissolving 20.0 mg of each crystalline pure powder in 10.0 ml methanol to achieve a primary concentration of 2.00 mg/ml. 5-Aminosalicylic acid (5-ASA) solution (20.0 μg/ml) was also prepared in methanol as an internal standard (IS). All the stock solutions were kept in capped glass tubes covered with aluminum foil and stored at 4 °C.

Working standard solutions were prepared by appropriate dilutions of the 2.00 mg/ml stock solutions to produce final concentrations of 0, 0.05, 0.10, 0.20, 0.50, 2.00, 10.0 and 500 μg/ml with the mobile phase, freshly before analysis. The standard samples for calibration curves were prepared by spiking the blank samples with the appropriate working solutions to yield a series of concentrations and the QC samples were prepared by spiking the analyte working standard solution into corresponding blank samples.

### 2.4. Animals

Sprague–Dawley rats ($240 \pm 10$ g, mean ± S.D.) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Animals were acclimatized for 1 week prior to experimentation in a temperature-controlled, 12/12 light/dark room, and were allowed for standard laboratory food and water. The rats were fasted overnight with free access to distilled, deionized water before the experiment. The study was conducted in compliance with standard animal use practices and was approved by Institutional Committee on Animal Uses at Purdue University.

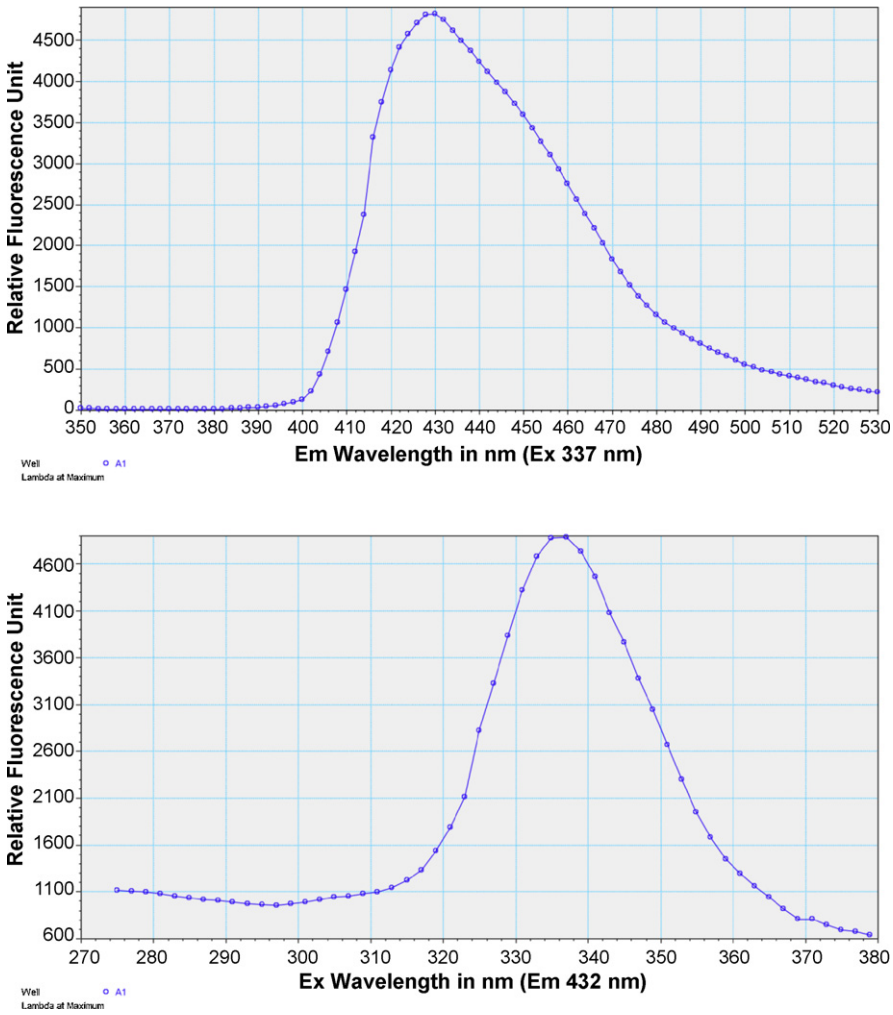


**Fig. 1.** Fluorescent spectra of PAS in mobile phase. For the collection of the characteristic fluorescent spectra, the scan was conducted with $\lambda_{exc}$: 275–378 nm and $\lambda_{emiss}$: 380–530 nm with 2 nm distance.

### 2.5. Plasma sample preparation

The plasma sample was thawed at room temperature before analysis. One volume (200 μl) of plasma was mixed with equal volume (200 μl) of the internal standard working solution to achieve final IS concentrations of 10.0 μg/ml. An aliquot of 300 μl methanol was added for protein precipitation and the sample pH value was adjusted to 1.0 by adding 20 μl of 6.0 M hydrochloric acid. After vortex mixing for 1 min, the suspension was centrifuged at 12,000 × *g* for 20 min using an Eppendorf centrifuge and the supernatant was dried under the nitrogen in the hood. Residue was dissolved in 150 μl of mobile phase, vortexed for 1 min, and centrifuged at 12,000 × *g* for 20 min. The supernatant was transferred to autosampler vial with sealed cap, and stored in a refrigerator at 4 °C prior to HPLC analysis within 1 day. An aliquot (10 μl) of the solution was injected into the HPLC for analysis. Plasma standard curves were constructed by diluting PAS and AcPAS standard stock solutions with blank rat plasma, to produce final concentrations of 0, 0.05, 0.10, 0.20, 0.50, 2.00, 10.0 and 500 μg/ml. Aliquots (200 μl) of these plasma standard samples received the same plasma treatment prior to HPLC analysis ($n = 5$).

### 2.6. Cerebrospinal fluid sample preparation

The CSF sample was thawed at room temperature before analysis, and mixed with an aliquot (200 μl) of the internal standard working solution; sample pH value was adjusted to 1.0 by adding 5.5 μl of 6.0 M hydrochloric acid, after which the sample was centrifuged at 12,000 × *g* for 5 min. The supernatant was dried under the nitrogen in the hood. All the following treatments were the same as described under Section 2.5. An aliquot (50 μl) of the solution was injected into the HPLC for analysis. CSF standard curves were constructed by diluting PAS and AcPAS standard stock solutions with artificial cerebrospinal fluid (aCSF) (consisting of 103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose and 1 mM sodium pyruvate at pH 7.4) to produce final concentrations of 0, 0.017, 0.033, 0.067, 0.167, 0.667, 3.33 and 166.7 μg/g. Aliquots (200 μl) of these CSF standards received the same CSF treatment prior to HPLC analysis ($n = 5$).

### 2.7. Brain sample preparation

The homogenized brain tissue samples or brain vasculature samples were thawed at room temperature before analysis, mixed with a volume (200 μl) of the internal standard working solution. For protein precipitation, an aliquot (300 μl) of methanol was added and the sample pH was adjusted to 1.0 by adding small volumes of 6.0 M hydrochloric acid. After standing for 15 min at room temperature, tissues were disrupted ultrasonically using Digital Sonic Dismembrator 500 (Thermo Fisher Scientific, Pittsburgh, PA), vortexed for 1 min, and centrifuged at 12,000 × *g* for 20 min. The

supernatant was transferred to a 0.45 μm centrifugal filter device (Pall, Port Washington, NY), filtered by centrifuging at 5000 × *g* for 10 min. The supernatant was then dried under the nitrogen in the hood. All the remaining treatments were the same as described under Section 2.5. An aliquot (50 μl) of the solution was injected into the HPLC for analysis. Brain standard curves were constructed by diluting PAS and AcPAS standards stock solutions with blank brain homogenate suspension, to produce final concentrations of 0, 0.017, 0.033, 0.067, 0.167, 0.667, 3.33 and 166.7 μg/g. Aliquot (200 μl) of these tissue standards received the same protein precipitating treatment prior to HPLC analysis ($n = 5$).

### 2.8. Capillary depletion

The capillary depletion process was carried out according to a well established procedure in this laboratory by Zheng et al. [25]. Briefly, brain tissues were homogenized in an ice-cold solution of buffer-I and dextran 70 (30%) in a ratio of 1:3:4 (brain:buffer-I:dextran 70). The buffer-I solution contained 2.603 g/l HEPES, 8.240 g/l NaCl, 0.298 g/l KCl, 0.174 g/l $MgSO_4 \cdot 3H_2O$, 0.138 g/l $NaH_2PO_4 \cdot 2H_2O$, and 0.399 g/l $CaCl_2$. The homogenate was centrifuged at 5400 × *g* for 15 min at 4 °C. The pellet was carefully separated from the supernatant. Light microscopic examination confirmed that the pellet consisted mainly of networks of brain vessels, while the supernatant was essentially depleted of vasculature. The pellet enriched with vasculature was washed with a mixture of 0.3 ml buffer-I and 0.4 ml dextran-70, and centrifuged once more to obtain the purified brain capillary. The pellet was placed in a disposable plastic tube and left uncapped in the ventilation hood for 12 h to the dryness, and frozen at −80 °C until analysis. The samples were processed within 3 days in the same way as described under Section 2.7.

### 2.9. PAS administrations and sample collection

There were six rats in each study group. PAS was dissolved in sterile saline each day prior to administration. Rats were anesthetized with ketamin/xylazine by i.p. injection. The femoral artery was surgically exposed and isolated; a small incision was made in the isolated artery. A polyethylene cannula was inserted into the incision and secured. For the rats in PAS treatment groups, 200 mg/kg of PAS was injected through the cannula, and rats in control groups received the same injections of sterile saline. CSF samples were obtained at 45 min after injection through a 26-gauge butterfly needle (Becton, Dickinson and Company, Franklin Lakes, NJ) inserted between the protuberance and the spine of the atlas, and were free of blood. Blood samples were collected from the inferior vena cava into a 2 ml heparinized syringe. Following standing at room temperature for 0.5 h, the blood was centrifuged at 3400 × *g* for 10 min using an Eppendorf centrifuge, after which an aliquot (200 μl) of the plasma was collected.

Rat brain was perfused with saline using a Heidolph pump drive PD 5201 system (Heidolph, Schwabach Germany), at a flow rate of 0.8 ml/min through the left common carotid artery. After 15 min of perfusion, the brain was dissected from the skull, washed with ice-cold saline, and then placed on an ice-cold filter paper. The choroid plexus was collected from lateral and third ventricles. Five other brain regions, including striatum, hippocampus, motor cortex, cerebellum and thalamus, were dissected in the order and separated into two parts. One half was used for brain regional capillaries depletion study, while the other half was homogenized using a homogenization buffer containing 20 mM Tris (pH 7.5), 5 mM EGTA, 1% Triton X-100, 0.1% SDS, 10 μl/ml PMSF, 15 mM 2-mercaptoethanol and a Protease Inhibitor Cocktail (Calbiochem, San Diego, CA) in a ratio of 1:3 (tissue: buffer). Rat CSF, plasma and brain homogenates were placed in disposable plastic tubes and immediately frozen at −80 °C until analysis. The samples were processed for extraction as described above within 3 days.

### 2.10. Method validation

The method validation was carried out according to bio-analytical method validation guidance announced by U.S. Food and Drug Administration (FDA) [26].

#### 2.10.1. Assay specificity

The specificity of the method was evaluated by analyzing blank samples from six individual rats in control groups. Each blank sample was tested for interference using the proposed preparation.

#### 2.10.2. Linearity and range

Calibration curves were constructed from the working solutions of PAS and AcPAS at a concentration range of 0.050–500.0 μg/ml in plasma, a concentration range of 0.017–166.7 μg/g in aCSF and brain tissue accordingly, by plotting the peak area ratio (*y*-axis) of PAS and AcPAS to the internal standard, 5-ASA, versus the corresponding PAS and AcPAS concentrations (*x*-axis). The regression parameters of slope, intercept and correlation coefficient were calculated by linear regression. The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve at which the accuracy (relative error, RE%) was within ±20% and the precision (relative standard deviation, RSD%) was below 15%.

#### 2.10.3. Precision and accuracy

The accuracy and precision of the analytical method were evaluated using QC samples injected into the HPLC for intra-day precision evaluation. The same procedure was performed for three consecutive days to determine the inter-day precision and accuracy. The concentration of each sample was determined using the calibration curve prepared and analyzed on the same batch.

#### 2.10.4. Recovery and stability

A recovery study was conducted by comparing the peak areas obtained from extracted samples spiked with known amounts of analytes (low, medium and high) with those obtained from the pure compounds of the same concentrations in mobile phase. For freeze and thaw stability study, the QC samples of plasma, CSF, brain at 3 concentrations were stored at the storage temperature (−80 °C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen under the same conditions for 12 h. The freeze–thaw cycles were repeated twice, and the samples were analyzed after three freeze–thaw cycles. For the long-term storage stability study, the QC samples at 3 concentrations were kept at storage temperature (−80 °C) were studied for a period of two weeks.

## 3. Results and discussion

### 3.1. Method validation

#### 3.1.1. Sensitivity and selectivity

During the method development, we took the advantage of the fluorescence detection for its high sensitivity and selectivity, coupling it with HPLC separation in order to eliminate the interferences present in plasma, CSF and brain tissues. From the fluorescence scans of both PAS and AcPAS, we found that the fluorescent response of AcPAS was much greater than that of PAS at the same molar concentration and also at the wavelengths with the maximum PAS absorbance. Thus, the excitation wavelength of 337 nm and emission wavelength of 432 nm were selected for the rest of studies (Fig. 1). 5-ASA was chosen as the internal standard

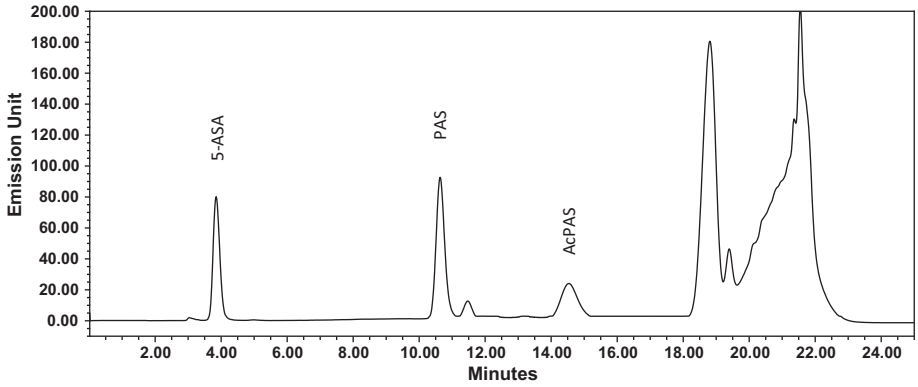


**Fig. 2.** Typical chromatogram of PAS, AcPAS, and 5-ASA which were added to blank brain samples from rats. No endogenous interferences were observed ($\lambda_{exc}$ = 337 nm; $\lambda_{emiss}$ = 432 nm). Retention time: 5-ASA = 3.85 min; PAS = 10.64 min: AcPAS = 14.54 min.

**Table 2**
Linear calibration correlation coefficients and LLOQ of PAS and AcPAS in body matrices ($n$ = 5).

| Curve | PAS | | | | AcPAS | | | |
|---|---|---|---|---|---|---|---|---|
| | Slope | Intercept | $R^2$ | LLOQ | Slope | Intercept | $R^2$ | LLOQ |
| Plasma | 291.8 ± 3.5 | 4.094 | 0.9991 | 50 ng/ml | 92.4 ± 0.4 | −1.699 | 0.9997 | 50 ng/ml |
| aCSF | 414.6 ± 0.8 | −3.666 | 1.0000 | 17 ng/g | 134.1 ± 0.2 | −5.360 | 0.9999 | 17 ng/g |
| Brain | 307.6 ± 3.4 | 3.095 | 0.9991 | 17 ng/g | 104.8 ± 0.5 | −8.068 | 0.9993 | 17 ng/g |

$R^2$: correlation coefficient of linear calibration curves. LLOQ: lower limit of quantification.

(IS) for its similarity with the major analytes in structure, chromatographic behavior, fluorescent behavior, and stability. In our preliminary study, we tested the direct HPLC–mass spectrometry as the method of choice for PAS quantification in blood and tissues. However, the severe interferences caused by the matrix effect in these studies, as well as the concerns on the high equipment cost and inconvenience in routine clinical analysis, prompted us to abandon this approach in the early stage of method development.

The selectivity of our method was evaluated by comparing plasma, CSF and brain chromatograms in the control group with those prepared by spiking the standard solutions into corresponding blank samples. PAS, AcPAS and the internal standard, 5-ASA were well separated under the present chromatographic condition with the retention times for PAS, 10.64 min, AcPAS, 14.54 min and 5-ASA, 3.85 min (Fig. 2). No endogenous fluorescent interferences were observed at or near the peaks of all 3 molecules, indicating a high selectivity of this method.

### 3.1.2. Linearity and detection range

This assay possesses a unique feature of simplified sample preparation procedure. Only one step of sample deproteination is required prior to direct HPLC analysis; the procedure avoids a complicated derivatization step commonly seen in HPLC analysis. The parameters of the linear calibration curves, derived from the statistical analysis of 3 independently prepared seven-point calibration curves in plasma, aCSF or brain and presented in Table 2, showed a good linearity of the assay for standards in these matrices. The plasma calibration curves had a correlation coefficient in the concentration range of 0.05 and 500 μg/ml. The LLOQ in plasma for both PAS and AcPAS were 50 ng/ml. The LLOQ of parent compound PAS in rat plasma by our study was about the half of the detection limit (94 ng/ml) reported by Gennaro et al. [20], who used the ion interaction reagent (IIR) for HPLC analysis of PAS.

The calibration curves for both PAS and AcPAS in brain homogenate and aCSF showed an excellent linearity in the concentration range of 0.017–166.7 μg/g of tissue or CSF weight. The LLOQ in brain and aCSF were found to be 17 ng/g (Table 2). This is the first method in the literature assaying PAS and its metabolite in brain tissues. Noticeably, our method is also the first in literature capable of quantifying both PAS and AcPAS simultaneously by HPLC.

### 3.1.3. Precision and accuracy

The results for method's intra- and inter-day accuracy and precision studies are presented Table 3 for both PAS and AcPAS in plasma or Table 4 for both analytes in aCSF and brain tissue. For intra-day and inter-day precision, RSD% values calculated for most of samples were between 1 and 8%. Detection of PAS and AcPAS in brain tissues at LLOQ showed a relative large variation (Table 4). The accuracy deviation values of both the analytes were between 93 and 109%. These data suggest an excellent consistence, precision and reproducibility of this method.

### 3.1.4. Recovery and stability

The absolute recoveries of PAS from plasma and brain homogenates were between 64–67% and 67–69%, respectively, while the absolute recoveries of PAS from aCSF were between 94 and 97% (Table 5). For AcPAS, the absolute recoveries from plasma, brain tissue and aCSF were between 65–66%, 77–85%, and 94–97%, respectively (Table 5). Among the factors that may influence the recovery (such as temperature, pH values, and pre-

**Table 3**
Intra- and inter-day precision (RSD%) and accuracy (RE%) of HPLC analysis of PAS and AcPAS spiked in plasma ($n$ = 5).

| Analytes | Spiked (μg/ml) | Plasma | | | |
|---|---|---|---|---|---|
| | | Intra-day | | Inter-day | |
| | | RE (%) | RSD (%) | RE (%) | RSD (%) |
| PAS | LLOQ | −6.1 | 7.6 | −9.3 | 11.5 |
| | 0.1051 | 8.9 | 3.44 | −1.5 | 3.21 |
| | 5.173 | 7.5 | 1.20 | −0.8 | 1.49 |
| | 254.8 | −5.0 | 1.02 | 0.7 | 1.85 |
| AcPAS | LLOQ | 7.9 | 5.4 | 10.7 | 8.2 |
| | 0.1051 | −3.4 | 4.85 | 0.8 | 4.9 |
| | 5.173 | 2.4 | 3.21 | −2.0 | 3.37 |
| | 254.8 | 4.1 | 2.45 | −0.4 | 4.57 |

**Table 4**
Intra- and inter-day precision (RSD%) and accuracy (RE%) of HPLC analysis of PAS and AcPAS spiked in brain tissues and aCSF ($n = 5$).

| Analytes | Spiked (μg/g) | Brain tissue | | | | aCSF | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Intra-day | | Inter-day | | Intra-day | | Inter-day | |
| | | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) |
| PAS | LLOQ | −16.7 | 11.3 | −18.4 | 13.8 | 4.5 | 2.72 | 3.9 | 3.87 |
| | 0.0781 | −5.8 | 4.36 | 1.4 | 7.42 | 4.0 | 2.57 | 5.3 | 3.11 |
| | 9.459 | −6.3 | 0.26 | −2.5 | 5.63 | −5.9 | 1.27 | 3.5 | 1.06 |
| | 155.2 | −0.3 | 0.39 | −0.2 | 4.81 | −4.9 | 0.38 | −0.9 | 1.15 |
| AcPAS | LLOQ | 12.4 | 6.73 | 17.1 | 9.61 | 2.3 | 1.77 | 3.0 | 2.67 |
| | 0.0781 | −5.5 | 2.24 | 6.5 | 3.24 | 2.5 | 1.68 | −4.1 | 3.56 |
| | 9.459 | −5.4 | 1.82 | −3.8 | 2.15 | −5 | 0.94 | −1.4 | 0.73 |
| | 155.2 | 6.0 | 0.73 | 1.3 | 2.41 | −6.2 | 0.52 | 0.3 | 1.29 |

**Table 5**
Absolute recoveries of PAS and AcPAS in plasma, aCSF and brain homogenates at three concentration levels ($n = 5$).

| Analyte | Sample | Spiked concentration | Mean (%) | RSD (%) |
|---|---|---|---|---|
| PAS | Brain | 0.078 (μg/g) | 69.2 | 5.16 |
| | | 9.459 (μg/g) | 66.9 | 2.33 |
| | | 155.2 (μg/g) | 67.1 | 1.60 |
| | aCSF | 0.078 (μg/g) | 96.0 | 1.89 |
| | | 9.459 (μg/g) | 97.4 | 0.96 |
| | | 155.2 (μg/g) | 94.1 | 0.74 |
| | Plasma | 0.105 (μg/ml) | 66.5 | 5.87 |
| | | 5.173 (μg/ml) | 64.4 | 1.05 |
| | | 254.8 (μg/ml) | 64.8 | 2.06 |
| AcPAS | Brain | 0.078 (μg/g) | 85.3 | 4.77 |
| | | 9.459 (μg/g) | 81.1 | 1.91 |
| | | 155.2 (μg/g) | 76.7 | 2.04 |
| | aCSF | 0.078 (μg/g) | 96.9 | 4.20 |
| | | 9.459 (μg/g) | 93.5 | 0.99 |
| | | 155.2 (μg/g) | 95.8 | 0.47 |
| | Plasma | 0.105 (μg/ml) | 64.6 | 5.02 |
| | | 5.173 (μg/ml) | 66.2 | 3.30 |
| | | 254.8 (μg/ml) | 66.5 | 2.56 |

Absolute recovery was calculated by comparing the peak areas obtained from extracted samples with those obtained from the pure compounds of the same concentrations in mobile phase.

cipitation reagents), we found that the pH value had a significant impact on the absolute recovery of both PAS and AcPAS. A series of recovery experiments were conducted by adjusting the samples to a series of pH values at 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0. The recovery of PAS decreased with increasing pH values, which was 22% at pH = 2.0 and increased to 81% at pH = 0.5 in brain homogenates. We also found that the recovery of AcPAS was 44% at the pH of 0.5 and a higher pH value appeared to yield a better recovery. Taking into account the recovery of both PAS and AcPAS, we chose the pH value at 1.0 for the sample preparation.

Studies on the freeze–thaw stability and the long-term stability revealed no significant decrease in concentrations of both PAS and AcPAS (Table 6). The results confirmed no stability-related problems during routine analysis of the samples.

### 3.2. Brain regional distribution of PAS and AcPAS

Our previous study suggests that treatment of Mn-exposed rats with subcutaneous injections of 200 mg PAS/kg effectively reduces Mn concentrations in blood, CSF, brain tissues and major organs examined [27]. To understand the mechanism of PAS action, there is a need to investigate the distribution of PAS and/or its metabolite in various brain regions. The assay developed in this study has made it possible to extract, identify, and quantify PAS and its major metabolite in plasma, CSF and selected brain regions following PAS administration. Our original study was focused on the distribution of PAS in brain. However, the HPLC chromatogram revealed a distinct peak eluded after the PAS peak. This finding prompted us to investigate the possible metabolite in brain tissues as a result of biotransformation of PAS. The literature search suggests AcPAS being a major PAS metabolite by acetylation of PAS [28]. Our current method with improved mobile phase and gradient profile was able to separate PAS from AcPAS in a single run in plasma (Fig. 3)

**Table 6**
Stabilities of PAS and AcPAS in rat brain tissue, aCSF and plasma at three concentration levels ($n = 5$).

| Matrices | Analyte | Concentration spiked | Long-term stability | | Freeze and thaw stability | |
|---|---|---|---|---|---|---|
| | | | 0 day | 14 days | Before cycle | 3 cycles of freeze–thaw |
| Brain | PAS | 0.078 (μg/g) | 103.4 ± 2.2 | 104.2 ± 2.3 | 104.4 ± 3.5 | 96.2 ± 5.7 |
| | | 9.459 (μg/g) | 102.0 ± 1.4 | 98.8 ± 6.1 | 107.7 ± 1.1 | 103.5 ± 11.5 |
| | | 155.2 (μg/g) | 100.6 ± 0.84 | 99.3 ± 1.4 | 101.5 ± 1.5 | 100.6 ± 5.3 |
| | AcPAS | 0.078 (μg/g) | 100.9 ± 2.1 | 102.0 ± 5.3 | 99.4 ± 1.5 | 96.8 ± 4.6 |
| | | 9.459 (μg/g) | 99.7 ± 3.0 | 100.6 ± 2.5 | 100.1 ± 1.8 | 97.4 ± 5.0 |
| | | 155.2 (μg/g) | 100.8 ± 2.8 | 100.2 ± 5.0 | 112.8 ± 2.4 | 103.1 ± 7.7 |
| aCSF | PAS | 0.078 (μg/g) | 103.3 ± 0.8 | 100.1 ± 2.8 | 99.9 ± 1.3 | 94.9 ± 3.9 |
| | | 9.459 (μg/g) | 96.2 ± 2.8 | 100.4 ± 2.3 | 105.4 ± 2.9 | 95.4 ± 1.4 |
| | | 155.2 (μg/g) | 99.3 ± 3.1 | 102.1 ± 2.1 | 111.6 ± 1.4 | 100.0 ± 3.6 |
| | AcPAS | 0.078 (μg/g) | 103.4 ± 1.6 | 105.2 ± 1.7 | 104.2 ± 3.1 | 97.8 ± 6.6 |
| | | 9.459 (μg/g) | 97.7 ± 1.1 | 100.3 ± 2.9 | 105.9 ± 1.9 | 95.3 ± 1.9 |
| | | 155.2 (μg/g) | 96.0 ± 2.3 | 99.9 ± 2.7 | 100.8 ± 0.84 | 94.9 ± 1.1 |
| Plasma | PAS | 0.105 (μg/ml) | 102.9 ± 4.1 | 97.2 ± 5.0 | 105.8 ± 3.0 | 95.2 ± 6.9 |
| | | 5.173 (μg/ml) | 103.4 ± 1.3 | 100.3 ± 2.6 | 108.6 ± 2.2 | 98.7 ± 6.1 |
| | | 254.8 (μg/ml) | 101.3 ± 3.6 | 99.3 ± 1.5 | 109.1 ± 3.1 | 101.7 ± 3.9 |
| | AcPAS | 0.105 (μg/ml) | 96.7 ± 3.8 | 101.0 ± 1.7 | 102.7 ± 2.8 | 105.2 ± 4.8 |
| | | 5.173 (μg/ml) | 98.6 ± 0.79 | 99.6 ± 1.8 | 110.3 ± 1.8 | 103.8 ± 7.2 |
| | | 254.8 (μg/ml) | 99.2 ± 2.1 | 99.9 ± 0.91 | 112.4 ± 3.5 | 106.8 ± 10.3 |

The long-term stability study was carried out by comparing concentrations of non-frozen QC samples with that kept at storage temperature (−80 °C) for two weeks. The freeze–thaw stability study was carried out by comparing concentrations of non-frozen QC samples with that went through three freeze–thaw cycles.

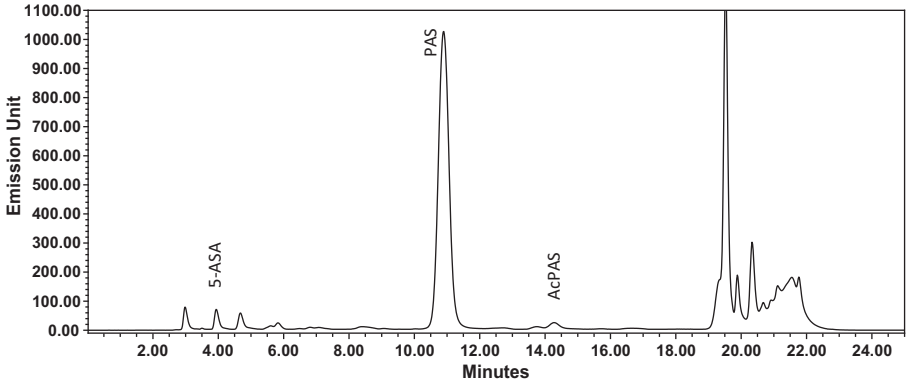


**Fig. 3.** Typical chromatogram of rat plasma following iv injection of PAS. Rats received iv injection of 200 mg PAS/kg; blood samples were collected at 45 min after injection and prepared for plasma. Samples were prepared for HPLC analysis as described in the main text. Retention time: 5-ASA = 3.94 min, PAS = 10.89 min, AcPAS = 14.28 min.

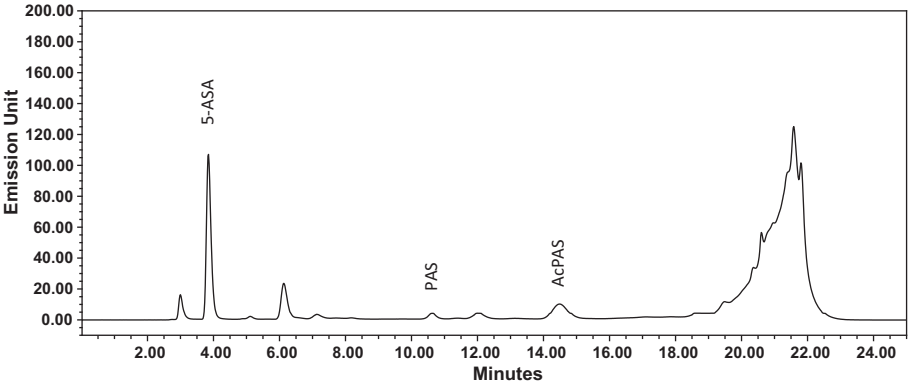


**Fig. 4.** Typical chromatogram of rat brain following iv injection of PAS. Rats received iv injection of 200 mg PAS/kg and brains were dissected at 45 min after injection. Samples were prepared for HPLC analysis as described in the main text. Retention time: 5-ASA = 3.85 min, PAS = 10.63 min, AcPAS = 14.49 min.

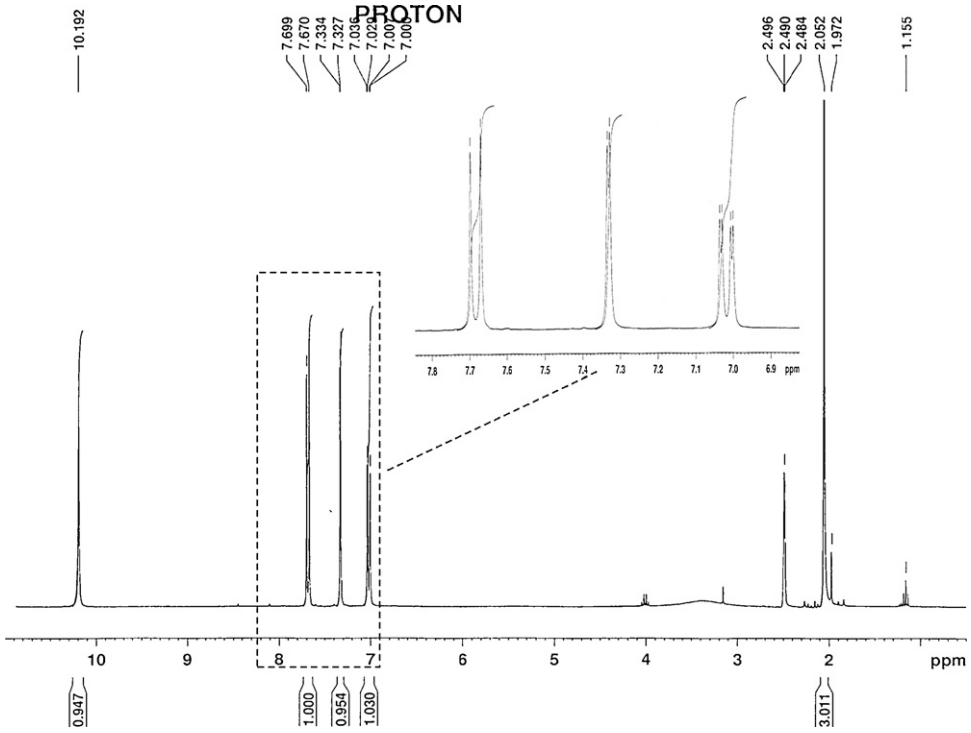


**Fig. 5.** $^{1}$H NMR spectroscopy to verify AcPAS in brain tissues. The HPLC fraction was collected between 14–15 min and subjected to MNR analysis (DMSO-$d_6$, 500 MHz). The spectra reveal the major species collected being correspondent to the structure of AcPAS.

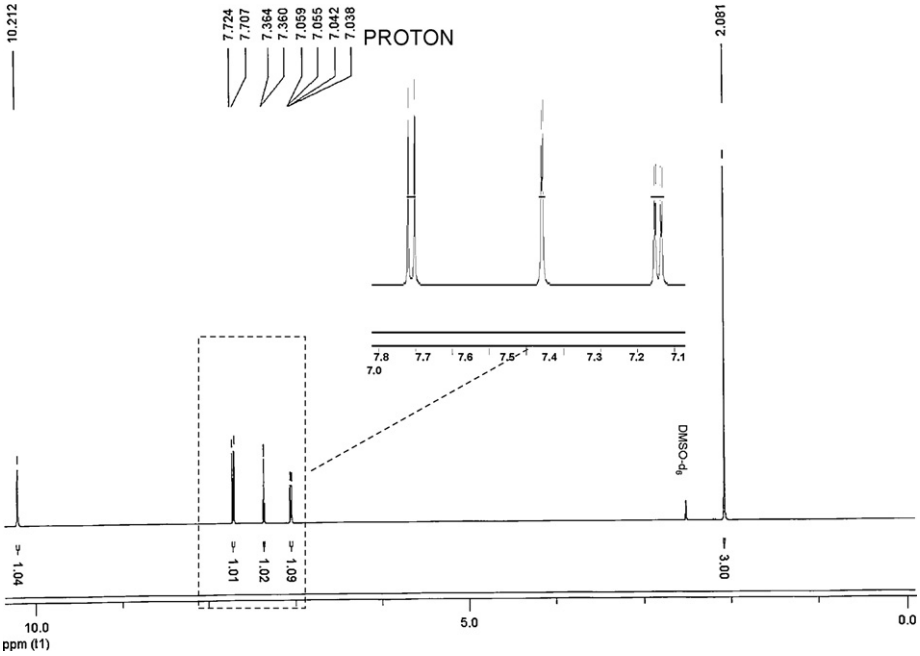


**Fig. 6.** $^1$H NMR spectra of the synthesized N-acetylated derivative (DMSO-$d_6$, 500 MHz).

and brain tissues (Fig. 4) collected from rats being injected with PAS. To verify that the peak at 14.49 min from rat brain (Fig. 4) was indeed AcPAS species, we collected the fraction between 14 and 15 min from the HPLC and determined the sample by the $^1$H NMR spectroscopy (Fig. 5). By comparing with the $^1$H NMR spectra of synthesized AcPAS (Fig. 6) and the data reported [19,29], it was clear that the major species in this fraction indeed corresponded to the structure of AcPAS. This proved that the method we developed can be used effectively to quantify both PAS and AcPAS in a single HPLC run; further the result suggests that following PAS systemic injection, both PAS and AcPAS can be detected in rat brain.

By using this method, we conducted a preliminary study to decide the optimal time point for tissue dissection and PAS analysis. The preliminary data showed that PAS and AcPAS had the $t_{max}$ around 45 min after iv injection of PAS. Hence this time point was used for tissue distribution study. The distribution profiles of the PAS and its metabolite in the brain regions are shown in Fig. 7.

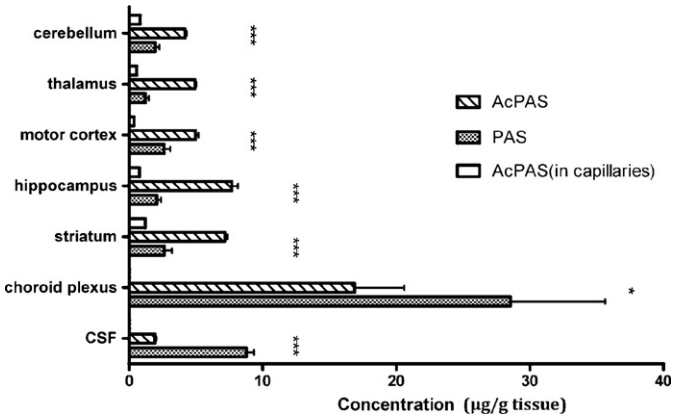


**Fig. 7.** Concentrations of PAS and AcPAS in cerebellum, thalamus, motor cortex, hippocampus, striatum, choroid plexus, and CSF. Rats received iv injection of PAS at a dose of 200 mg/kg; 45 min after injection, brains were dissected and analyzed for PAS and AcPAS using the HPLC method described in the main text. Data represent mean ± S.D., $^{***}p < 0.0001$, $^{*}p < 0.05$, $n = 6$.

Following iv injection, PAS and AcPAS distribution in brain and CSF showed the following characteristics: (1) parent PAS was higher than AcPAS in the CSF and choroid plexus (a barrier between the blood and CSF); (2) among brain regions analyzed, the metabolite AcPAS was generally higher than PAS; (3) AcPAS, but not parent PAS (which was below the detection limit), was detected in brain capillary fraction, which forms the blood–brain barrier; and (4) both PAS and AcPAS were much higher in the choroid plexus than those in the CSF, brain capillary, and parenchymal tissues. The concentrations of PAS and AcPAS in rat plasma at 45 min after dosing were 225.4 ± 14.1 μg/ml and 37.38 ± 3.93 μg/ml, respectively, indicating that the parent, unchanged PAS molecules were the predominant species in the blood circulation. Interestingly, in both brain parenchyma and capillary, the metabolite AcPAS, rather than the parent PAS, was the major species following PAS injection. This observation suggested that PAS was metabolized to AcPAS either by cerebral endothelial cells that constitute the blood–brain barrier or by brain cells in targeted area, or both.

Our data raise several interesting questions. First, what is the effective species (PAS or AcPAS) that binds with Mn and removes the metal from the brain regions where the metal accumulates? Second, where and how is PAS metabolized to AcPAS, in brain barrier cells or neurons or other neuroglial cells? Third, what are the relative roles of the blood–brain barrier in cerebral endothelia verses the blood–CSF barrier in the choroidal epithelia in PAS or its metabolite's penetration to brain? Finally, by which route, i.e., the blood–brain barrier or blood–CSF barrier, does the PAS remove Mn from the cerebral compartment? Answers to these questions will significantly advance our understanding of the mechanism by which PAS alleviates the Mn-induced parkinsonian syndromes and help develop a novel strategy for treatment of Mn neurotoxicity. Perceivably, the newly developed HPLC method in this report shall play an indispensable role in the due course.

## 4. Conclusion

A new HPLC method with one-step protein precipitation and rapid sample preparation was established for determination of PAS and its main metabolite, AcPAS, in rat plasma, CSF and brain tissues.

The method has been validated for its excellent precision, sensitivity, selectivity and accuracy on these three matrices. Application of this method to quantify PAS and AcPAS in rat brain following a single iv administration of PAS showed a greater amount of AcPAS than PAS in brain tissues. The data support a practical use of this method to quantify PAS and AcPAS in plasma, CSF and tissues.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.11.031.

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