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Is alumina suitable for solid phase extraction of catecholamines from brain tissue?

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Occupational and environmental toxicology specialists find catecholamine fluctuations in brain tissue relevant for research of neurotoxicity, such as that induced by manganese or zinc, pesticides, industrial solvents, plastic, air pollution, or irradiation. Considering that catecholamine tissue concentrations are generally very low, their extraction requires a reliable and optimal method that will achieve maximum recovery and minimise other interferences. This study aimed to evaluate whether the aluminium (III) oxide (Al_2O_3, alumina) based cartridges designed for catecholamine isolation from plasma could be used for solid-phase extraction (SPE) of catecholamine from the brain tissue. To do that, we homogenised Wistar rat brain tissue with perchloric acid and compared three extraction techniques: SPE, the routine filtration through a 0.22 μm membrane filter, and their combination. In the extracts, we compared relative chromatographic catecholamine mobility measured with high performance liquid chromatography with electrochemical detection. Chromatographic patterns for norepinephrine and epinephrine were similar regardless of the extraction technique, which indicates that the alumina cartridge is good enough to isolate them from brain tissue. However, the dopamine pattern was unsatisfactory, and further experiments are needed to identify the issue and optimise the protocol.

KEY WORDS: catecholamines; aluminium oxide; solid phase extraction; brain; tissue

Catecholamines is the common name for three biogenic amine compounds: dopamine, norepinephrine, and epinephrine. Their common precursor is L-tyrosine, which undergoes hydroxylation and decarboxylation to yield dopamine. Further hydroxylation metabolises dopamine into norepinephrine, which forms epinephrine after receiving the methyl group. The enzymes essential for catecholamine metabolism are monoamine oxidase and catechol-*O*-methyl transferase. Besides their physiological role as neurotransmitters, catecholamines are involved in the normal functioning of the cardiovascular system and regulation of metabolism. In the clinical context, high catecholamine concentrations in the plasma and urine may indicate catecholamine-secreting tumours such as pheochromocytoma. In addition, catecholamine levels are being investigated in different biological fluids as potential biomarkers of psychiatric, neurodegenerative, and cardiovascular conditions (1, 2).

Catecholamines are essential in brain physiology, as they participate in the neural regulation of various processes like food uptake, movement coordination, memory, or sleep-wake cycles (3, 4). Their involvement in the pathogenesis and treatment of neurological conditions such as Parkinson's and Alzheimer's disease, depression, or epilepsy has also gained much interest among researchers (3, 5, 6). In addition, pharmacological assessments have identified a complex link between anxiety and immunomodulation under stress conditions (7), and various toxicology disciplines analyse brain catecholamines to evaluate the neurological effects of exposure to heavy metals like manganese, lead, or zinc (8–10). Experimental studies modelling the neurotoxicity effects of pesticides, industrial solvents, or plastics also rely on data about brain catecholamine levels (11, 12), and knowing these levels has helped to establish a dose-dependent relationship between irradiation dose and brain function impairment (13). It has become clear that for brain research catecholamine analysis in brain tissue has a substantial advantage over plasma (2, 14).

However, the concentrations of catecholamines are low whether in plasma or brain, which calls for sensitive and accurate measurement. The current state-of-the-art in catecholamine analytics includes methods based on electrochemical sensors or biosensors, spectroscopy, and separation techniques required for high-performance liquid chromatography (HPLC) and capillary

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electrophoresis coupled with traditional detectors (ultraviolet, fluorescence, or electrochemical) or mass spectrometers (15). Due to an optimal balance between analytical performance (specificity, sensitivity, and reproducibility), cost, and operational challenges, HPLC is the usual method of choice (2, 16). However, its performance largely depends on the efficiency of catecholamine extraction, which, in turn, depends on catecholamine abundance and stability in (brain) tissue and the interference of the matrix and other compounds. Sample clean-up protocols for biological fluids utilise various solid phase extraction (SPE) techniques, and one of the most common and the longest in use employs aluminium (III) oxide (Al_2O_3, alumina) as an extraction medium. However, protocols for catecholamine extraction from tissues have not involved SPE, even though other extraction methods could introduce artefacts in chromatographic separation (1). This is why we wanted to see how well would SPE using alumina-based cartridges fare in catecholamine extraction from brain tissue, as we believed that the method would lower the risk of interferences, and uniform extraction procedure would bring additional reliability in measuring catecholamine levels. To test its reliability we, therefore, compared the results of SPE with routine filtration and a combination of SPE and filtration.

MATERIALS AND METHODS

The study included three adult (8–10-week-old) male Sprague Dawley rats, weighing 180-250 g, bred at the experimental animal facility of the Belgrade University Faculty of Pharmacy (Belgrade, Serbia) and maintained under standard laboratory conditions (temperature 21±2 °C, relative humidity 40–45 %, and illumination 120 lx) with free access to pellet food and tap water. The animals were kept on a 12:12 h light/dark cycle with lights on at 06:00 h. All handling took place during the light phase of the diurnal cycle. The rats were anaesthetised with an intraperitoneal dose of ketamine hydrochloride (90 mg/kg, Ketamidor, Richter Pharma AG, Wels, Austria) and xylazine hydrochloride (10 mg/kg, Xylased, Bioveta, A. S., Ivanovice na Hane, Czech Republic), then euthanised with carbon dioxide (17), and their brain tissue samples (20–150 mg) from the frontal lobe collected on ice.

The study complied with the EU Directive 2010/63/EU (18) and was approved by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia − Veterinary Directorate (Decision No. 323-07-09943/2018-05).

Ice-cold 0.1 mol/L perchloric acid (Merck, Darmstadt, Germany) was added to the tissue samples (5 μL/mg). After 10 min of incubation on ice, vortexing, and centrifugation at 16,000 g for 10 min (Megafuge 16®, Thermo Fisher Scientific, Waltham, MA, USA), the supernatants were pooled to minimise variation in brain catecholamine content between the experimental animals. The pooled extracts were then purified in three different ways. The first was direct purification with the alumina cartridge, the second was filtration through a 0.22 μm membrane filter (Costar® Spin-X®, Corning, USA), and the third combined filtration and purification with the alumina cartridge.

For the alumina-based purification we used the ClinRep® HPLC Complete Kit for catecholamines in plasma (Cat. No. 1000, Recipe, Munich, Germany) according to manufacturer's instructions. As an internal standard we added a solution of 3,4-dihydroxybenzylamine (DHBA) included in the kit (Cat. No. 1012), which had to be purified by filtration only.

After purification, we injected a volume of 40 μL into the HPLC system (Waters Corporation, Milford, MA, USA) consisting of a 2695e Alliance® separation module and a 2465 electrochemical detector (ECD). The reversed-phase (particle size 5 μm) HPLC column (2) included in the kit (Cat. No. 1030, Recipe) was used for isocratic chromatographic separation with the mobile phase (Cat. No. 1210, Recipe) flow of 1 mL/min, as recommended by the manufacturer. The mobile phase contained 50 mmol/L of potassium dihydrogen phosphate, 2.5 mmol/L of 1-octanesulfonic acid sodium salt, 0.1 g/L of ethylenediaminetetraacetic acid/acetonitrile (96.5:3.5, v/v), pH 3.5, and phosphoric acid (2). The ECD settings were: range 2 nA/V, filter 2S, and DC mode of the voltage supply. For chromatographic data collection and processing we used the Empower 3® software (Waters).

The quality of chromatographic separation quality was assessed by analysing the commercial standard solution for catecholamines in plasma (ClinTest® Standard Solution, Cat. No. 1011, Recipe). For norepinephrine and epinephrine, the manufacturer reports the linearity range (LR) 15-2500 ng/L, lower limit of detection (LLOD) 8 ng/L, and the lower limit of quantification (LLOQ) 15 ng/L. For dopamine the LR is 50-2500 ng/L, LLOD 15 ng/L, and LLOQ 30 ng/L. Depending on the concentration, the intra-assay precision is 5.1-8.8 % for norepinephrine, 5.9-10.9 % for epinephrine, and 4.2-8.6 % for dopamine. The inter-assay precision for norepinephrine, epinephrine, and dopamine is 4.2-6.5 %, 3.8-4.8 %, and 2.7-5.9 %, respectively. The recovery is 70-90 %.

RESULTS AND DISCUSSION

Figures 1–5 present characteristic chromatograms obtained in the study, including the peak heights and the areas under the peaks. Figure 1 shows the chromatogram of the DHBA solution, and Figure 2 the results for the ClinTest® Standard Solution.

Figures 3–5 show representative chromatograms of brain catecholamine extracts purified using either the alumina cartridge, routine filtration, or their combination. Alumina purification alone or in combination with filtration resulted in chromatograms without the dopamine peak (Figure 3 and 5, respectively), whereas filtration only showed all three catecholamine peaks (Figure 4).

As retention times for catecholamines and internal standard varied with batches, we calculated relative retention time (RT_r) for each chromatogram by dividing the retention time of the internal standard by the retention time of each catecholamine (Table 1). The

A

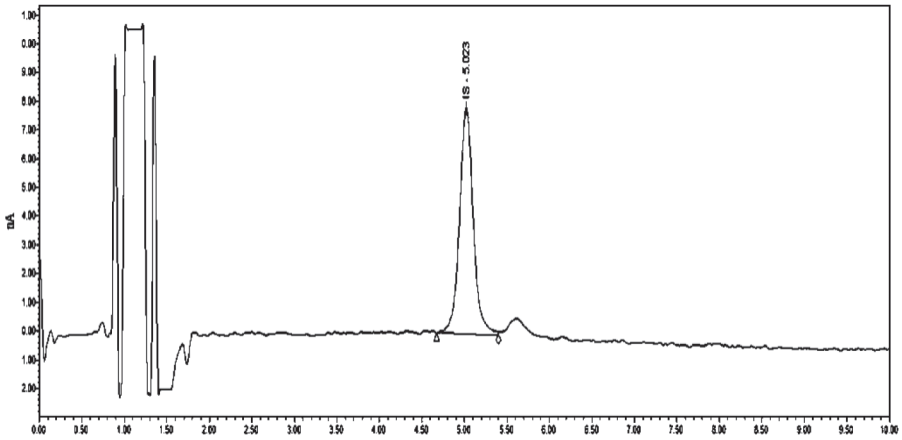

Figure 1 A) Chromatogram of the 3,4-dihydroxybenzylamine solution, used as the internal standard (the number above the component is the retention time in minutes); B) Identified component with peak height and the area under the peak; IS – internal standard

B

Peak	Area	Area (%)	Height
IS	17228550	100.00	1563699

A

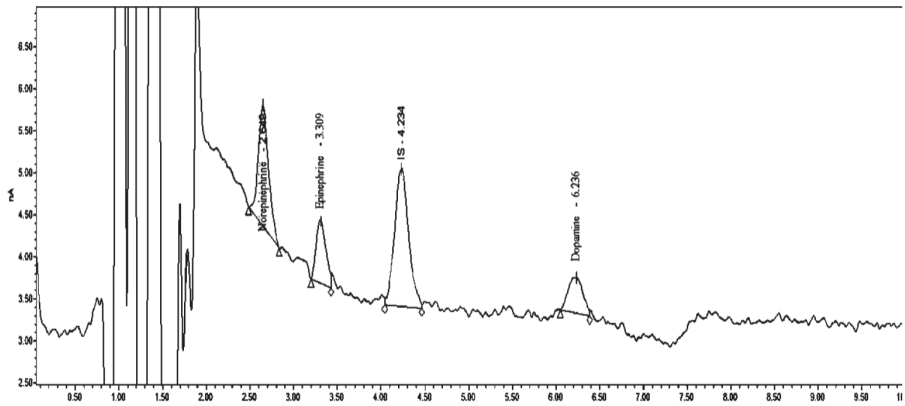

Figure 2 A) Chromatogram of the ClinTest® Standard Solution (the number above the component is the retention time in minutes); B) Identified components with peak height and the area under the peak; DP – dopamine; E – epinephrine; IS – internal standard; NE – norepinephrine

B

Peak	Area	Area (%)	Height
NE	2341612	29.33	287249
E	1206555	15.11	147906
IS	3336295	41.79	314984
DP	1098753	13.76	88511

A

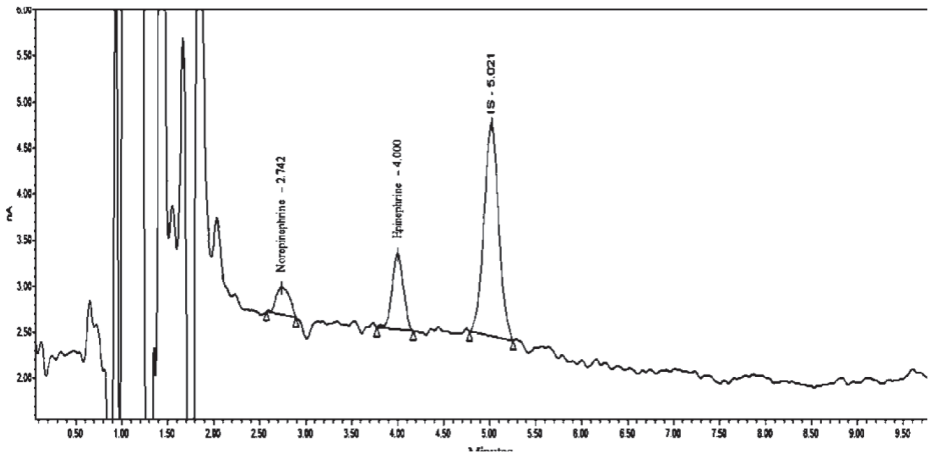

Figure 3 A) Chromatogram of the extract purified on the alumina cartridge (the number above the component is the retention time in minutes); B) Identified components with peak height and the area under the peak; E – epinephrine; IS – internal standard; NE – norepinephrine

B

Peak	Area	Area (%)	Height
NE	614034	8.55	61719
E	1356991	18.90	166417
IS	5208003	72.54	472773

A

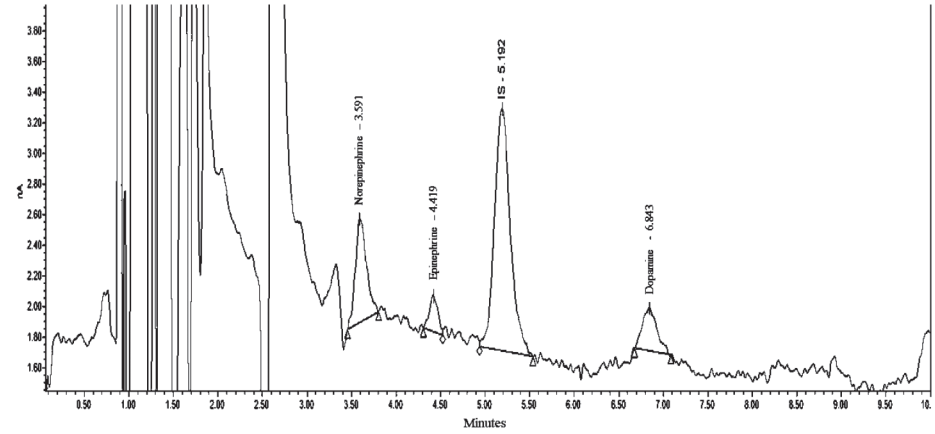

Figure 4 A) Chromatogram of the extract purified by filtration (the number above the component is the retention time in minutes); B) Identified components with peak height and the area under the peak; DP – dopamine; E – epinephrine; IS – internal standard; NE – norepinephrine

B

Peak	Area	Area (%)	Height
NE	942915	15.68	121421
E	265684	4.42	43070
IS	3769653	62.68	311894
DP	1035303	17.22	72678

A

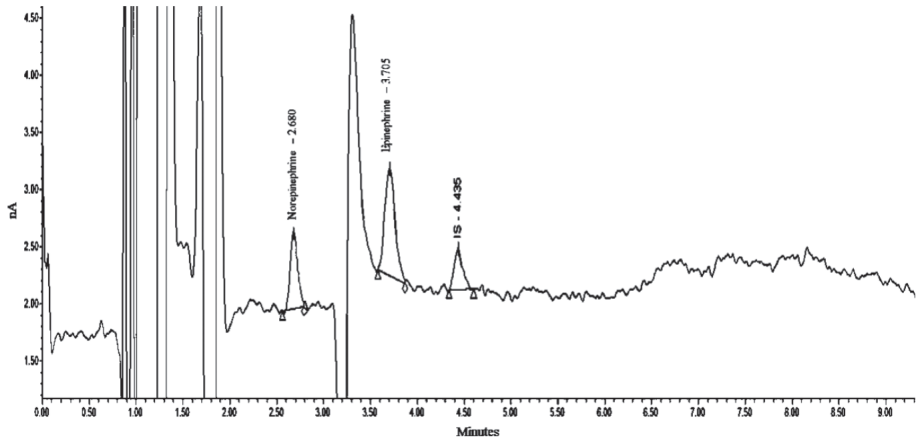

Figure 5 A) Chromatogram of the filtrated extract purified on the alumina cartridge (the number above the component is the retention time in minutes); B) Identified components with peak height and the area under the peak; E – epinephrine; IS – internal standard; NE – norepinephrine

B

Peak	Area	Area (%)	Height
NE	696631	27.34	130434
E	1395625	54.78	186378
IS	455655	17.88	73506

Table 1 Relative retention times of norepinephrine, epinephrine, and dopamine by extract purification method

Solution	RT_r		
	NE	E	DP
IS	/	/	/
ClinTest® standard	1.657	1.279	0.679
Extract purified on alumina	1.831	1.255	/
Filtered extract	1.445	1.175	0.759
Filtered extract purified on alumina	1.654	1.197	/

DP – dopamine; E – epinephrine; IS – internal standard; NE – norepinephrine; RT_r – relative retention time

result was acceptable uniformity, as norepinephrine and epinephrine had similar RT_r in the ClinTest® Standard Solution and all extracts. Dopamine, in turn, showed similar uniformity in ClinTest® Standard Solution and the extract obtained by filtering alone.

Our preliminary results show that alumina has acceptable SPE properties for norepinephrine and epinephrine determination in brain tissue with HPLC with EC detection and point to two issues. The first is the absence of dopamine peaks on chromatograms of extracts prepared using SPE, either alone or after filtration (Figures 3 and 5). The second is the occurrence of an artificial peak on the chromatogram of the extract obtained by combining SPE and filtration (Figure 5). We cannot provide an explanation (e.g. suboptimal buffering during the SPE on alumina) of these issues at this point, and leave to further studies to identify the reasons.

Even so, our study clearly shows the potential of SPE, originally designed for catecholamine analytics in plasma and urine, to be used for brain tissue. Mirković et al. (19) have already successfully validated Bio-Rex 70C®, a weak cation exchange resin, as an SPE matrix for catecholamine extraction from rat adrenal, kidney, thymus, spleen, and hypothalamus tissue. Our findings show that a mechanism based upon cyclic structure formation between alumina and the catechol moiety (20) also holds the potential to isolate norepinephrine and epinephrine from the tissue extract. Notwithstanding the limitations, these pilot results encourage further research which should compare the efficiency of different SPE matrices and identify the conditions yielding optimal catecholamine recovery.

The SPE properties of alumina-based commercial cartridges confirmed in our study substantially extend the range of possibilities for their practical use. Moreover, our findings raise further questions about the advantages of alumina-based SPE over filtration, such as, could the commercial DHBA solutions included in the assay kits for plasma catecholamine be used to *spike* the brain tissue extracts to resolve recovery issues?

The limitations of our study are mostly owed to the pilot study design and small number of samples, for which reason the results lack traceable calibration and quality control specific for the (brain) tissue extract. This limitation can be addressed with additional assessments and optimisation of extraction conditions as a starting point for large-scale analytical and clinical validation studies.

To conclude, alumina as the SPE matrix proved satisfactory for norepinephrine and epinephrine extraction from rat brain tissue, even with a protocol employing perchloric acid for tissue homogenisation. The evident absence of dopamine peak on chromatograms is a potential disadvantage associated with alumina

use for this purpose and will need to be addressed by further research.

Acknowledgements

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Conflicts of interest

None to be declared.

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Je li aluminijev oksid pogodan za postupak ekstrakcije čvrste faze katekolamina iz moždanoga tkiva?

Promjene razine katekolamina (KAT) u moždanom tkivu značajne su za brojna područja profesionalne toksikologije odnosno ekotoksikologije u kojima se istražuje neurotoksičnost izazvana različitim agensima poput mangana ili cinka, pesticida, industrijskih otapala, plastike, aerozagađenja ili zračenja. Niske koncentracije KAT-a u tkivu zahtijevaju pouzdanu i učinkovitu tehniku ekstrakcije kojom se postiže maksimalni „prinos" katekolamina i minimalni sadržaj interferirajućih spojeva. Cilj istraživanja bio je procijeniti mogu li se nosači na bazi aluminijeva (III) oksida (Al_2O_3), dizajnirane za izolaciju KAT-a iz plazme, koristiti za ekstrakciju čvrstom fazom (eng. solid-phase extraction – SPE) KAT-a iz moždanoga tkiva. Nakon homogenizacije tkiva Sprague Dawley štakora upotrebom perklorne kiseline, primijenjene su tri tehnike ekstrakcije: SPE, filtracija kroz 0,22 µm membranski filtar, koji je zapravo rutinska tehnika za izolaciju KAT-a iz mozga, i kombinacija tih dviju tehnika. U ekstraktima je relativna kromatografska pokretljivost KAT-a analizirana HPLC metodom s elektrokemijskom detekcijom. Ponašanje norepinefrina i epinefrina tijekom kromatografije bilo je slično, bez obzira na tehniku ekstrakcije, što upućuje na to da aluminijev oksid ima zadovoljavajuća svojstva izolirati ta dva KAT-a iz moždanoga tkiva. Međutim, uočeni su problemi s ekstrakcijom dopamina, koji zahtijevaju dodatne eksperimente kako bi se otkrio njihov uzrok i osmislio protokol optimizacije.

KLJUČNE RIJEČI: aluminijev oksid; ekstrakcija čvrste faze; katekolamini; mozak; tkivo