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A useful procedure for detection of polyamines in biological samples as a potential diagnostic tool in cancer diagnosis

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Abstract

Background: Polyamines present in human body are frequently considered as markers of occurrence of cancer. Therefore, the availability of simple and efficient method for determination of their level in body liquids and tissues is of some interest.

Methods: Supported liquid membrane technology coupled with HPLC seems to be an appropriate technique to follow the level of polyamines in human blood and urine. Thus, the membranes of two different geometries: flat sheet and hollow fiber were studied as a mean for separation and enrichment of studied polyamines from urine and tissue samples in order to prepare samples to be analyzed by HPLC.

Conclusions: Developed extraction systems offer an interesting alternative to traditional techniques such as: liquid-liquid or solid-phase extraction due to several features, which are: very high enrichment of polyamines without previous work-up, simple procedure of extraction and tiny volume of organic solvents used. This enables efficient determination of their levels in body liquids.

Keywords: Spermine, Spermidine, Cadaverine, Putrescine, Supported liquid membranes, Cancer markers

Background

Naturally occurring polyamines-spermine, spermidine, cadaverine and putrescine, are widespread in the nature and play a crucial role in processes of cell growth, differentiation, development and death [1–3]. Although their biochemistry and physiologic role is not well understood it was shown that they are implicated in growth and development of neoplastic cells [4–6]. Recently, their *N*-acetyl derivatives detected in urine were demonstrated to be of clinical use as markers of lung cancer [7, 8]. Thus, their elevated level may serve as non-specific tumor markers, especially for the pre-cancer diagnosis [4, 9–13]. Their level could be also used for the monitoring efficacy of therapy [9, 10]. Disappointingly, polyamines are difficult to analyze because they are small polar compounds, occurring in complex biological samples and usually in small quantities. Unfortunately they also are easily oxidized upon work-up of biological samples. Therefore, the efforts were undertaken in order to design and evaluate analytical methods for determination and quantification of these amines [12, 14].

The whole analytical process of biological sample consists of: sample collection, sample preparation, and detection of analyzed compounds followed by data analysis. Sample preparation is, as a rule, the longest, the most laborious and the most expensive stage. It can absorb even 80% of time and cost of the whole analysis [15, 16]. Therefore, in this paper we have studied the use of liquid membranes (SLMs), immobilized in porous polymeric matrices of two different shapes (flat sheet and hollow fiber ones), for separation and enrichment of natural polyamines from urine and tissue samples prior to their analysis by standard HPLC technique. The use of membrane technology is a promising alternative to

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commonly used liquid-liquid extraction and solid phase extraction (SPE).

Methods

Polyamine hydrochlorides and derivatizing agent-tosyl chloride, were purchased from Fluka (Buchs, Switzerland). All salts, acids and bases used for preparation of the buffers and adjusting the pH of the donor and acceptor phases were of analytical grade and were obtained from POCH (Gliwice, Poland). Acetonitrile of HPLC grade was obtained from Merck (Darmstadt, Germany). Water was purified with Milli-Q-RO4 system (Millipore, Bedford, MA, USA).

Liquid membrane solvent - dihexyl ether, and membrane carrier - di-(2-ethylhexyl)phosphoric acid (D2EHPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fluoropore FG flat circular sheet membranes of 9 cm diameter (porosity 0.7, pore size-0.2 μm, thickness115 μm) were purchased from Milipore. Q3/2 Accurel PP. Hollow fiber membrane in a shape of capillary with 0,6 mm inner diameter and 200 μm wall thickness (0,2 μm average pore size) was obtained from Membrana (Wuppertal, Germany).

HPLC system used for determination of polyamines consisted of Ultimate 3000 series modules (Dionex, Sunnyvale, CA, USA): vacuum degasser, gradient pump, autosampler with 50 μL loop working in the partial injection mode (20 μL of sample was injected) and diode array detector. Analytes were separated on Gemini C-18 column (Phenomenex, Torrance, CA, USA) with 5 μm average diameter of particles and 4,6 mm × 250 mm dimensions. Chromatographic data were collected and analyzed using Chromeleon 6.08 software (Dionex).

Tissue collection

Tissue samples were collected from four patients who were operated at The First Department and Clinic of General, Gastroenterological and Endocrinological Surgery of Wrocław Medical University. All patients were euthyroid and had a normal level of thyroid-stimulating hormone (TSH). They were not treated with any drugs before the surgery. Thyroid specimens (the tumor tissues and the healthy tissues from the second, healthy thyroid lobe that is routinely resected in such situations) were collected intraoperatively. Each sample was snap frozen in liquid nitrogen and stored at −80 °C. Histological assessment and classification of these tumors as carcinoma papillare were conducted according to the criteria of the WHO [17].

Preparation of donor phases

The 5 mM stock solution of polyamines was prepared using degassed water and polyamine hydrochlorides. The solution was degassed with a stream of nitrogen and divided into portions that were next frozen (to prevent oxidation of amines). The portions were stored in a freezer up to 3 weeks and were thawed before usage.

Fresh, morning urine of healthy person was adjusted to desired pH using 1 M HCl or solid KOH under a control of pH-meter (Beckman, Fullerton, USA) and supplemented with polyamine after pH adjustment.

100 mg of porcine kidney or human thyroid tissue was homogenized with 0.5 mL 10% trichloroacetic acid and 0.5 mL water in 1 mL Wheaton glass homogenization vessel (Milville, NJ, USA). Then the sample was centrifuged for 15 min (10 000 rpm). 1 mL of supernatant was taken and its pH was adjusted to 7 using 5 M KOH under the control of pH meter with very thin electrode ERH-13–6 (Hydrometr, Gliwice, Poland). Then the electrode was rinsed with water to gather any remaining analytes. The neutralized sample was subjected to SLM extraction. Depending on experiment, some of the samples were supplemented with polyamines before tissue homogenization and some after pH adjustment.

Supported liquid membrane extraction

All extractions were conducted in at least three repetitions. Error of the results is represented by standard deviation or by coefficient of variation (CV).

The flat sheet supported liquid membrane was prepared by immersing Fluoropore FG for several seconds in organic solvent consisting of 20% v/v D2EHPA in dihexyl ether. After impregnation membrane was rinsed with water and placed in an extraction unit described earlier [16]. The extraction module consisted of four cylindrical blocks of 12 cm diameter and 1 cm thickness. Supported liquid membrane was place between two inner blocks made from PTFE that one surface had grooved channel (0.25 mm deep, 1.5 mm wide and 2.5 m long) arranged as Archimedes spiral. Each channel had volume of 0.94 mL. The PTFE blocks were clamped by two aluminum blocks to make the construction more rigid and stable and were tightened with six screws. Sample (10 mL of studied solution) was pumped through the channels of the module using peristaltic pump Minipuls 3 (Gilson Medical Electronics, Villiersle-Bel, France). Diluted hydrochloric acid was used as acceptor phase.

Q3/2 Accurel PP hollow fiber was cut into 51 mm long pieces. One side of each fiber was melted by soldering tool and closed using forceps. To remove any impurities the prepared fibers were immersed in acetone, and dried. A lumen of the fiber was filled with acceptor phase (13 μl) using 25 μl HPLC syringe. The filled fiber (still attached to the syringe needle) was immersed in membrane organic liquid for 3–5 s in order to impregnate the pores of polypropylene support and thus to make SLM. Then, the fiber was rinsed with water to remove the excess of the liquid membrane phase.

Finally, the fiber was taken out from syringe needle and put onto soft wire (tin-lead-silver alloy, 0.7 mm diameter). Membrane could be used immediately for extraction or could be stored for 2–3 h in the beaker filled with water. During extraction the whole membrane was immersed in the sample placed in 2.0 mL donor phase (0.1–1.0 M HCl) in Eppendorf test tube. Extraction vessels were shaken using orbital shaker TTS2 at 600 rpm (IKA Werke, Staufen, Germany). After extraction, the fiber was taken out from the sample, dried with soft paper tissue and emptied with the use of an HPLC syringe filled with the air. The acceptor phase was pushed out into PCR tube (0.2 mL) and analyzed by means of HPLC.

Derivatization and HPLC analysis

Derivatization was performed according to the published procedure with some modifications. Thus, to the sample (5 μL) placed in 100-μL glass vessel equal volume of saturated solution of disodium carbonate, four volumes of potassium borate buffer (0.5 M, pH 11) and four volumes of derivtizing reagent (5 mg/mL of tosyl chloride in acetonitrile) were added. The samples were shortly vortex-mixed and left for 10 min. Afterward, one volume of 1 M hydrochloric acid was added, samples were stirred again and subjected to HPLC analysis. Water and acetonitrile were used as mobile phase while the elution program was maintained in dependence to sample matrix. The basic binary elution program was set up starting from 62% of acetonitrile up to 85% in 15 min. The flow rate of mobile phase was 1 mL/min and the detection wavelength was set on 229 nm.

Calculations

The extraction parameters that were used in this paper are enrichment factor and extraction efficiency. The enrichment factor (Ee) was calculated from Eq. 1:

$$E_e = \frac{C_A}{C_D^0} \quad (1)$$

where CA is the concentration of analyte in the acceptor phase after SLM extraction and C is the initial concentration of analyte in the donor phase (sample).

The extraction efficiency (E) was calculated from Eq. 2:

$$E = E_e \times \frac{V_A}{V_D} \times 100 = \frac{C_A V_A}{C_D^0 V_D} \times 100 \quad [\%] \quad (2)$$

where VA is volume of the acceptor phase and VD is volume of the donor phase.

Results and discussion

In order to improve the method for the determination of polyamines in real samples the usefulness of liquid membranes of two different geometries for enrichment and analysis of polyamines in two matrixes (urine and tissue extract) had been determined. Preliminary studies have shown that the modification of the conditions worked-out in previous studies [18] enabled to determine the level of these amines in urine and tissue homogenates.

Extraction of polyamines from urine

Due to the availability of urine in large volumes the application of flat sheet membrane module may be considered as more suitable than small hollow fiber membranes. Thus, a model system consisting of urine spiked with putrescine (Ptr), cadaverine (Cdr), sperimidine (Spd) or spermine (Spm), at final concentrations of 50 μM, was used (the donor phase). Previously the efficiency of this system for extraction of polyamines from non-buffered aqueous solutions was found to be independent on pH of the donor phase [19]. However, taking into account the fact that urine is reach in inorganic salts and therefore may act as some kind of a buffer, we set two pH values of donor phase at 4 and 7 using, standardly, 0.1 M hydrochloric acid as an acceptor phase. Indeed, urine components impede extraction of polyamines with extraction efficiencies being about two times lower from urine than from water (Fig. 1a). It can be explained on the basis on transport mechanism since apart from polyamines, D2EHPA being an acid is also able to transport other cationic substances present in urine such as inorganic salts and urea or creatinine. Moreover, their concentrations are much higher than concentrations of polyamines. Thus, they compete with polyamines for the membrane carrier significantly decreasing the flux of polyamines.

This could be overcame by increase of acidity of acceptor phase do pH 1 (Fig. 1b), which provides a higher pH gradient between acceptor and donor phases and consequently higher driving force of the transport. The application of donor phase of pH 7 appeared to be of choice yielding substantial improvement of extraction efficiency over this received from water. This effect could be explained by buffering capacity of urine. It is known that as a result of transport mechanism protons are released in donor phase. When using water this effect is quite substantial and the decrease of pH from 7 to 3.6 was observed upon the process whereas pH of urine stays unchanged during whole process. Of curse, to increase the pH gradient across the membrane pH higher than 7 of donor phase might be used, however, in this case the supported liquid membrane is not stable . This is because the carrier-D2EHPA forms salts, which are acting as surfactants and break down liquid membrane.

The enrichment of analytes is one of the most important tasks in sample preparation, especially in case of analysis of substances present in trace quantities. In

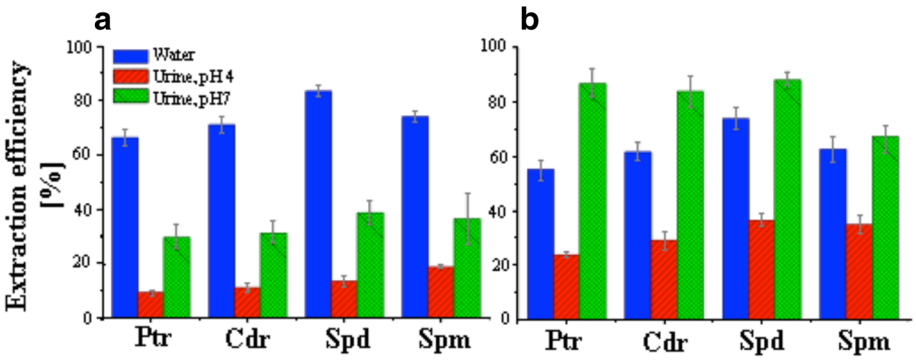

Fig. 1 Extraction efficiency of putrescine (Ptr), cadaverine Cdr), spermine (Spm) and spermidine (Spd) form urine by flat sheet supported liquid membrane in dependence of pH of the acceptor phase: (**a**) 0.1 M HCl as acceptor phase, (**b**) 1 M HCl as acceptor phase. Concentration of polyamines in donor phase is 50 μM

most SLM extraction systems enrichment factor (Ee) relies directly proportional to the volume of the analyzed sample. Thus, another mean to improve the efficiency of extraction is to increase the volume of the donor phase. We studied the polyamines enrichment factors after extraction of 10, 20 and 40 mL of urine (Table 1) and as expected the Ee values are higher the larger is the donor phase volume. Surprisingly, their increase is not so big as it could be expected from Eq. 2. Regarding the obtained results 20 mL seems to be optimal volume of donor phase. The extraction efficiencies are high and achieved enrichment factors are satisfying (6.9–8.5). Although the enrichment factor values are highest for 40 mL, the extraction times were also two times longer (3 h versus 20 min) and repeatability was much worse. Therefore, the optimal conditions of this analysis using flat sheet membrane module were set as: 20 mL of donor phase of pH 7; 20% v/v D2EHPA in dihexyl ether as membrane phase, 2 mL of 1 M hydrochloric acid as acceptor phase and 100 min of extraction with donor flow of 0.2 mL/min.

Previous attempts to apply hollow fiber technique to analyze polyamines in urine gave unacceptable results [19]. However, basing on the above-described results we have modified this approach by using 1 M HCl as an acceptor phase and by setting the time of extraction at 30 min. It is important because the application of longer extraction procedure, albeit resulting in higher enrichment of polyamines, also gave less repeatable results. Possible cause of that is nearly complete neutralization of acceptor phase after longer time of analysis. Anyway, the observed extraction efficiencies of putrescine, cadaverine, spermidine and spermine were 30.2, 33.5, 32.6 and 21.6% respectively, whereas corresponding enrichment factors were 46.4, 51.5, 50.1 and 33.2, which may be considered as satisfactory.

Table 1 The extraction efficiency (E) and enrichment factor (Ee) values of polyamines after flat sheet supported liquid membrane extraction from different volume of urine samples. The pH of urine was 7 and acceptor was 1 M HCl. Donor phase polyamines concentration was 50 μM. CV – coefficient of variation

Amine	Sample volume								
	10 ml			20 ml			40 ml		
	E [%]	E_e	CV [%]	E [%]	E_e	CV [%]	E [%]	E_e	CV [%]
Ptr	86.7	4.3	6.7	81.5	8.1	3.4	63.2	12.6	22.0
Cdr	83.8	4.2	7.0	79.5	7.7	5.4	63.5	12.7	17.5
Spd	88.1	4.4	3.6	86.4	8.5	5.2	78.1	15.6	10.2
Spm	67.4	3.4	5.8	70.3	6.9	6.4	73.8	14.8	7.8

HPLC analysis of SLM extracts from unspiked urine or spiked with very low concentration of polyamines evidenced that some other substance co-eluted with polyamines. Anyway, it ddi not affect the results of analysis and the elaborated method, especially proper selection of gradient program enabled efficient separation of all four polyamines (Fig. 2).

Extraction of polyamines from tissue samples

This part of the study was designed in hope that evaluation of concentration of polyamines in samples of the tissue taken by thin needle biopsy could be of help in preliminary diagnosis of cancer. Regarding the small mass and volume of the tissue only hollow fiber extraction may be applied.

To study and optimize the extraction process of analysis of polyamines in solid tissue homogenates we have chosen easily available porcine kidney tissue treating it as a model one. Before liquid membrane extraction step tissue samples were subjected to the homogenization with trichloroacetic acid solution. It was found that the kidney tissue homogenate contains quite high content of spermidine and spermine (tri- and tetraamine), and low of putrescine, cadaverine (which are diamines) (Fig. 3). Thus, single hollow fiber extraction step was enough to

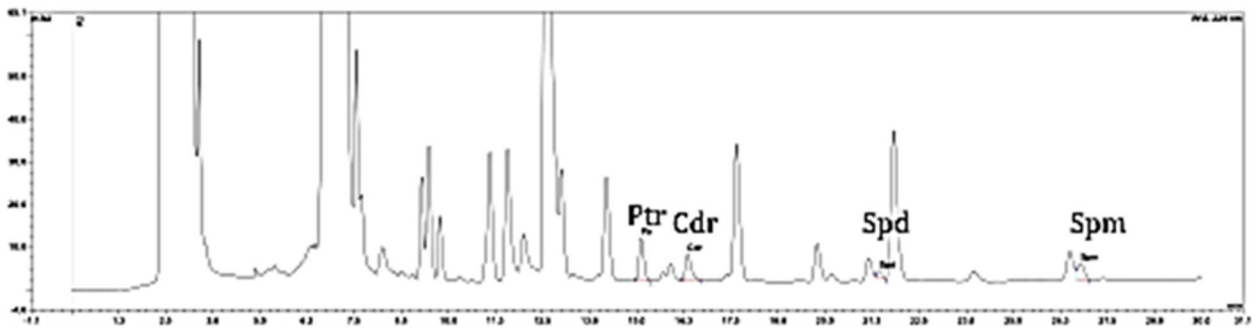

Fig. 2 Single hollow fiber supported liquid membrane extract of polyamines from unspiked urine of healthy person. Symbols of amines are as in Fig. 1. The elution program was convex gradient from 20% acetonitrile in water to 80% in 30 min

sample purification prior HPLC analysis. However, application of 1 M HCl as the acceptor phase negatively influenced transport kinetics, even if it is leading to more efficient extraction. Consquently, application of 0.5 M HCl and prolonging the time of extraction to 45 min allowed obtaining reasonable results. Finally, the optimal conditions of this analysis using hollow fiber membrane module were set as: 2 mL of donor phase of pH 7, stirring at 600 rpm; 20% v/v D2EHPA in dihexyl ether as membrane phase, 13 μL of 0.5 M hydrochloric acid as acceptor phase and 45 min of extraction.

Developed method was used to compare the content of polyamines present in healthy thyroid and malignant tumor (carcinoma papillare) tissues. The most important diagnostic tool for thyroid nodules is the cytological examination of the samples obtained by fine-needle aspiration biopsy [20]. However, retrospective studies of this diagnosis show the its limitations; the nodules with associated indeterminate or suspicious cytology represent 10–20% of cases with approximately 15% of all biopsies being assessed as insufficient for diagnosis [21]. Thus, the supporting tool is required to shape the diagnosis. The polyamine identification could be such a tool. Measurements of the level of polyamines were done on the basis of retention time and UV spectrum. In the case of three

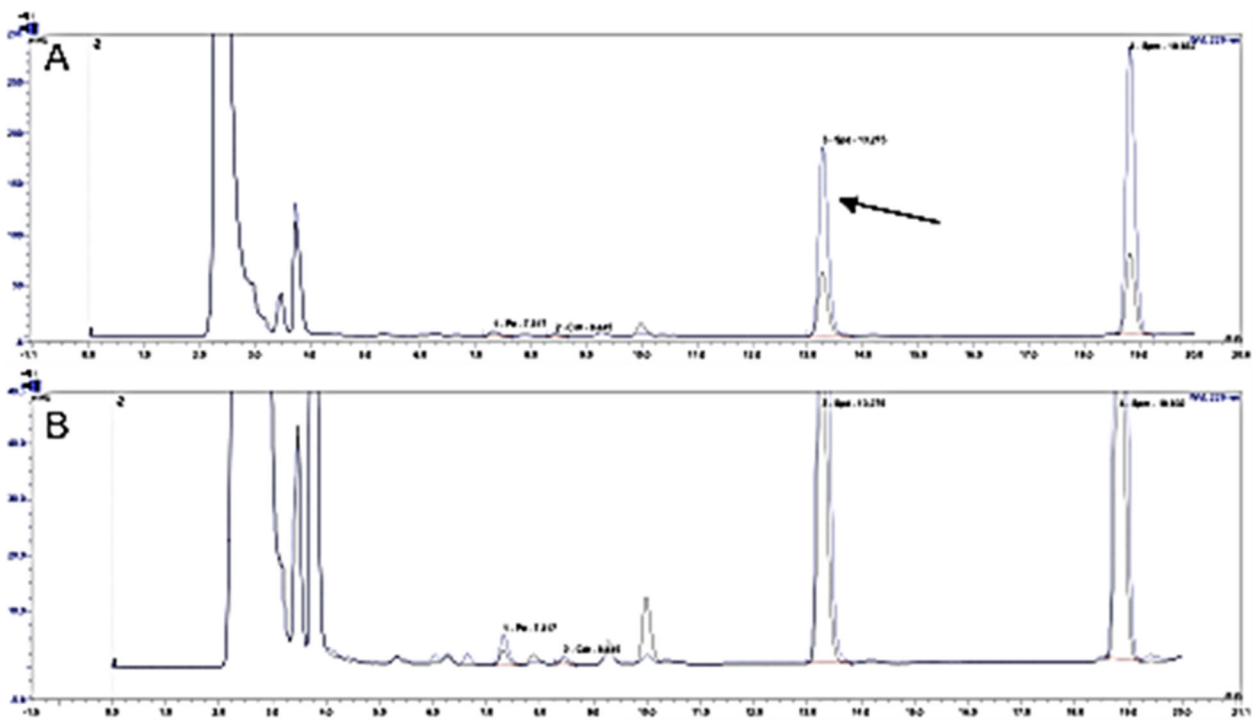

Fig. 3 Unspiked porcine kidney homogenate (**a**) and its supported liquid membrane extract polyamines (**b**) studied by HPLC. Extraction conditions: 0.5 M HCl as an acceptor phase and extraction time - 45 min

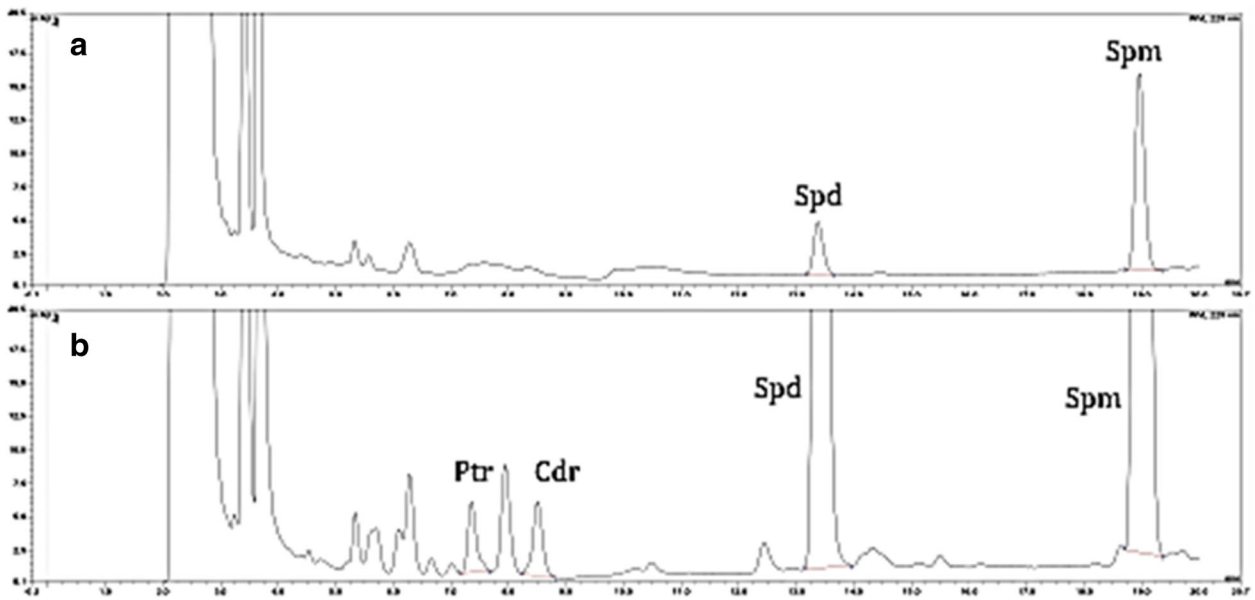

Fig. 4 Single hollow fiber supported liquid membrane extracts of polyamines from unspiked normal (*black* chromatogram) and tumor transformed thyroid tissue of the same patient (*blue* chromatogram indicated by arrow) coming from the representative patient with thyroid cancer (carcinoma papillare): (**a**) chromatogram and (**b**) its magnification

out of four studied patients the content of studied polyamines in tumor tissue was higher that in healthy tissues taken from the same patients (Fig. 4). This result, although promising, requires far detailed studies with the use of bigger set of samples needed for calibration (for exact quantification of polyamines) and validation of the procedure (for determining accuracy and precision of the measurements).

Conclusions

Determination of polyamines in urine, hair and human tissues is being considered as an additional method, which might be used to support classical cancer diagnosis [9–12]. The most important diagnostic tool for thyroid cancer is cytological examination of the samples obtained by fine-needle aspiration biopsy. However, application of this method has some shortcomings. Therefore search for new tools for supporting this diagnosis are of some value. Determination of the level of polyamines could serve here. Preliminary studies on the elaboration of the effective procedure for the use of liquid supported membrane technology to determine spermine, spermidine, cadaverine and putrescine and their relative ratios in urine and tissue homogenates indicate that this method could be considered as supportive diagnostic tool. However, the implementation of this method in clinics required additional extensive studies.

Abbreviations

Cdr: Cadaverine; D2EHPA: di-(2-ethylhexyl)phosphoric acid; HPLC: High performance liquid chromatography; Ptr: Putrescine; SLM: Supported liquid membrane; Spd: Sperimidine; Spm: Spermine; TRH: Thyroid-stimulating hormone; WHO: World Health Organization

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Availability of data and material

All source analytical data are available from Prof. Piotr Wieczorek, University of Opole.

Authors' contributions

WB: study conception and writing and editing of paper, tissue collection and storage, KP: data acquisition and performing of analyses, PW: design of analytical part od studies and manuscript editing. All authors approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All patients had access to the best available diagnostic methods and treatments according to actual scientific evidence and were not involved in any experimental method. The dignity, privacy, and confidentiality of personal information were guarantied throughout the study by codification and deleting all personal data that could make identifiable individual people. The protocol for this study was approved by the Commission of Bioethics at

the Wrocław Medical University (Approval no. KB-248/2010), and written informed consent was obtained from all the patients before enrollment in the study.

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