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Conversion of L-tryptophan to serotonin and melatonin in human melanoma cells

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Abstract We showed in human melanoma cells tryptophan hydroxylase (TPH) and hydroxyindole methyltransferase genes expression with the sequential enzymatic activities of TPH, serotonin (Ser) *N*-acetyltransferase and hydroxyindole methyltransferase. The presence of the products Ser, 5OH-tryptophan, *N*-acetylserotonin, melatonin (Mel), 5-methoxytryptamine and 5-methoxytryptophol was documented by liquid chromatography–mass spectrometry. Thus, human melanoma cells can synthesize and metabolize Ser and Mel. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Melanoma cell; Serotonin; Melatonin; *N*-Acetylserotonin

1. Introduction

The neurotransmitter serotonin (Ser) is produced through a multistep metabolic pathway starting with the hydroxylation of L-tryptophan (Trp) by tryptophan hydroxylase (TPH: EC 1.14.16.4) to hydroxytryptophan, followed by decarboxylation by an aromatic L-amino acid decarboxylase (EC 4.1.1.28) to Ser [1,2]. In the brain, pineal gland and retina Ser is acetylated by arylalkylamine *N*-acetyltransferase (AANAT: EC 2.3.1.87) to *N*-acetylserotonin (NAS), which is further methylated by hydroxyindole-*O*-methyltransferase (HIOMT: EC 2.1.1.4) to produce melatonin (Mel) [3].

Melanocytes are cells of neural crest origin that, whether normal or malignant, share the expression of a characteristic metabolic pathway, melanogenesis, which involves production of melanin. This is the result of a complex, multistep process that starts with the hydroxylation of L-tyrosine [4]. Mel has significant actions on melanocyte function, since it can inhibit melanogenesis (at pharmacologic concentrations) or melanoma proliferation (at physiologic concentrations) (reviewed in [5]). The Mel precursor, Ser, has been detected in malignant and normal human melanocytes (reviewed in [5]), and we reported that hamster skin can metabolize Ser to NAS and possibly to Mel [6,7]. In the present investigation, we have determined the full expression of the melatoninergic system in melanoma cells.

2. Materials and methods

2.1. Chemicals

All reagents used for enzymatic assays and for high-performance liquid chromatography (HPLC) separations were of the highest purity. L-[5-^{3}H]Trp was from Amersham Life Sciences Inc. (Arlington Heights, IL, USA). L-Trp, 5-OH L-Trp (5-OHTrp), Ser, NAS, Mel, 5-methoxytryptamine (5MTT), 5-methoxytryptophol (5MTOL), *N*-acetyltryptamine and tryptamine were purchased from Sigma (St. Louis, MO, USA). All chemicals for biochemical assays not listed above, including dye reagents for protein assay, were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

SKMEL188 human melanoma cells were grown in Ham's F10 medium supplemented with 10% fetal bovine serum and antibiotics (Gibco-BRL, Gaithersburg, MD, USA) as described previously [8]. Under the above conditions the cells maintain amelanotic phenotype [8]. Melanoma cells were detached using Ca and Mg free Tyrode's solution containing 1 mM EDTA and used for biochemical or molecular assays.

2.3. Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from melanoma cells using Trizol isolation kit (Gibco-BRL). The synthesis of first-strand cDNA was performed using the Superscript preamplification system (Gibco-BRL). 5 μg of total RNA per reaction was reverse-transcribed according to the manufacturer's protocol using oligo(dT) as the primer. All samples were standardized by the amplification of housekeeping gene GAPDH as described previously by Robbins and Mc Kinney [9]. Primers were synthesized by Integrated DNA Technology Inc. (Coralville, IA, USA).

PCR amplifications were performed under standard conditions with modifications where indicated. The reaction mixture (25 μl) contained 2.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.4 μM of each primer, 75 mM Tris–HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20 and 0.25 U of Taq polymerase (Promega, Madison, WI, USA). The mixture was heated to 94°C for 2.5 min and then amplified for 35 or 30 cycles as specified, cycles were as follows: 94°C for 30 s (denaturation), 65°C for 45 s (annealing) and 72°C for 1 min (extension).

Amplification of human TPH cDNA. Primers P100 and P101 were used in the first round of amplification. Sequences of primers were as follows: P100 5′-AGCCAGATACCTGCCATGAAC-3′; P101 5′-GCTGCAGCTCATTCATGGCAC-3′. An aliquot of PCR mixture from the first round of amplification was transferred to a new tube and a second round of PCR was conducted. Primers for the second round of PCR were: P106 5′-CCAAGAAATTGGCTTGGCTTCTC-3′; P107 5′-TGCTCTTGGTGTCTTTCAGGATC-3′.

Primers for the first round of human HIOMT gene amplification

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were: P118 (5′-TGAGAGAAGGAAGGAACCAGTAC-3′) and P119 (5′-CAAGTGTGGTAGATCCTCTCCAG-3′); and for the second round: P120 (5′-GAAGAGCTTTTTACGGCCATCTAC-3′) and P121 (5′-CGGAAGAGGGTCTTTGAAGAAATC-3′).

Identified PCR products were excised from the agarose gel and purified by GFX PCR DNA and gel band purification kit (Amersham-Pharmacia-Biotech). Sequencing was performed in the Molecular Resource Center at the University of Tennessee HSC (Memphis, TN, USA) using an Applied Biosystems 3100 Genetic Analyzer and BigDye™ Terminator kit.

2.4. Enzymatic assays

The radiometric assay for TPH was performed by Dr. Wilfred Pinto, according to the methodology described by Beevers et al. [10] with minor modifications. Melanoma cells or rat brain were homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.6) containing 2 mM dithiothreitol (DTT) in order to maintain the enzyme in the active conformation. The crude cell homogenate was utilized as the source material for the TPH assays. The assay mixture was composed of 50 mM Tris buffer containing 2 mM DTT, 100 μM L-Trp, ~10 nM L-[5-^{3}H]Trp as radioactive tracer (~100 000 cpm, 31 Ci/mmol), 0.5 mM 6-methyl-5,6,7,8-tetrahydropterine and crude cell homogenate containing 30–50 μg protein in a final volume of 200 μl. Adding the crude cell homogenate to the prepared assay mixture started the reaction. In control (background tubes) the cell extracts were substituted with an equivalent volume of assay buffer. Following incubation for 120 min at 37°C, the reactions were stopped by adding 40 μl of 60% perchloric acid ($HClO_4$) to the assay mixture to the concentration of 12%, and incubating for an additional 30 min at 37°C. Assay mixtures were then mixed with 250 μl of a Norit-Dextran T70 slurry (50 mg each/ml H_20); the samples were vortexed and centrifuged and the supernatants were used for liquid scintillation spectrometry in a Beckman LS7000 spectrometer.

AANAT activity was measured by a modification of the method described by Thomas et al. [11]. Cells were homogenized in an ice-cold 0.25 M potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 1 mM EGTA, protease inhibitor cocktail (2 μl/ml homogenization mixture) and 0.625 mM acetyl coenzyme A (CoA). Homogenates were centrifuged at 15 000×*g* for 10 min at 4°C. To measure enzymatic activity aliquots of 80 μl of supernatant were mixed with 20 μl of 5 mM Ser or tryptamine in 0.25 M potassium phosphate buffer (pH 6.8). The final concentrations of acetyl CoA and amine substrate were 0.5 mM and 1 mM, respectively. The mixtures were then incubated for 1 h at 37°C, and the enzymatic reaction was stopped by the addition of 20 μl of 6 M $HClO_4$. After centrifugation in a microcentrifuge at 4°C, the supernatant (30–35 μl) was subjected to HPLC in a system equipped with a Novapak C_{18} reverse-phase column (100×5 mm, i.d.) and fluorometric detector (Waters). The detector was calibrated with excitation and emission wavelengths set at 285 and 360 nm, respectively. The elution was carried out isocratically at ambient temperature with a flow rate of 1.5 ml/min for the different mobile phases depending upon the amine substrate used. The mobile phase contained 4 mM sodium 1-octanesulfonate as ion-pairing agent, 50 mM ammonium formate (pH 4.0) versus methanol (80:20) for Ser and (75:25) for tryptamine. Elution peaks of NAS and *N*-acetyltryptamine were identified by retention time (RT). Their identity was verified by their co-eluting with authentic standards. Quantitation was based upon calibration curves constructed from directly injected standard compounds (NAS and *N*-acetyltryptamine) and the concentration of the test samples was determined by interpolation. For background controls, the reaction mixture was incubated either without substrates or without an enzyme source.

HIOMT activity was assayed by a modification of the method described in [12], which is based on HPLC separation and fluorometric detection of Mel formed enzymatically from NAS and *S*-adenosyl-L-methionine. Briefly, melanoma cells were homogenized in an ice-cold 0.05 M sodium phosphate buffer (pH 7.9) containing 0.625 mM *S*-adenosyl-L-methionine. Homogenates were centrifuged at 15 000×*g* for 10 min at 4°C. Aliquots of 80 μl of supernatant were mixed with 20 μl of 5 mM NAS in 0.05 M sodium phosphate buffer (pH 7.9). The final concentrations of *S*-adenosyl-L-methionine and NAS were 0.5 mM and 1 mM, respectively. The mixtures were then incubated for 1 h at 37°C, and the enzymatic reaction was stopped by adding 20 μl of 6 M $HClO_4$. After centrifugation in a microcentrifuge at 4°C, the supernatant (60 μl) was subjected to HPLC analysis in the system described above for the measurement of AANAT activity with tryptamine as substrate. Elution peaks of NAS and Mel were identified by RT. Their identity was verified by their co-eluting with authentic standards and quantitation was conducted as described above. For background controls, the reaction mixture was incubated either without substrate or without an enzyme source. Protein concentration was determined by a dye-binding method with bovine serum albumin (BSA) as the standard [8].

2.5. Immunohistochemistry

Melanoma cells grown on glass coverslips were fixed in 4% buffered paraformaldehyde, washed with 50 mM Tris–HCl buffer (pH 7.6) and then incubated for 10 min in 30% v/v methanol:Tris buffer containing 20 μl of 30% H_2O_2 to block endogenous peroxidase. The non-specific binding was blocked by incubation for 1 h in Tris buffer containing 10% normal goat serum. The coverslips were then incubated for 18 h at room temperature with anti-Ser (Diasorin Corp., Stillwater, MN, USA) and anti-TPH sera (Chemicon, Temecula, CA, USA) diluted 1:5000 and 1:1000, respectively, with Tris buffer containing 1% BSA and 0.25% Triton X-100. After washing the coverslips were incubated with biotin-conjugated goat anti-rabbit polyclonal antibody (Vector Laboratories, Burlingame, CA, USA), and the presence of the primary antibody–secondary antibody complex was revealed by the avidin–biotin peroxidase complex method (Vector Elite kit, Vector Laboratories). The immune complexes were visualized using diaminobenzidine tetrahydrochloride as the chromogen.

2.6. Liquid chromatography–mass spectrometry (LC/MS)

The melanoma samples were analyzed by LC/MS using a Agilent 1100 Series LC/MSD SL mass spectrometer with atmospheric pressure interface–electrospray (API–ES) interface and with Agilent 1100 Series HPLC system with a high pressure binary pump (Agilent Technologies Inc., Wilmington, DE, USA), in the tandem mode. Briefly, SKMEL188 melanoma cell pellets were suspended in 0.1 N HCl, sonicated, centrifuged for 10 min at 15 000×*g* and supernatants were filtered through a Millex-LH filter (0.45 μm pore size). Aliquots of 2 μl were separated on a Agilent 1100 Series System through a Zorbax Eclipse XDB-C_{18} column (1.0 mm ID×150 mm L, 3.5 μm particle) with a mobile phase A: water with 7% methanol and 0.1% trifluoroacetic acid (TFA), and mobile phase B: methanol with 0.1% TFA. Separation was performed with methanol at the following gradients: 7% (1–12 min), 23% (12–15 min), 35% (15–20.1 min) and 7% (20.1–26 min), while maintaining the flow rate at 0.08 ml/min. The effluent from the HPLC system was routed to the MS through API–ES with selective ion monitoring (SIM) for *m/z* 177 (0–5.5 min), 221 (5.5–7 min), 217 (7–15 min), 205 (15–16.5 min), 191 (16.5–19 min) and 192 (19–23 min). The spray chamber conditions were as follows: gas temperature: 300°C; drying gas: 9 l/min; nebulizer pressure: 40 psig; V_{Cap} (positive): 3500 V. The corresponding standards were similarly analyzed by LC/MS under the same conditions.

3. Results

3.1. Enzymatic activities

Direct biochemical assays for enzymatic conversion of Trp to hydroxytryptophan showed high activity in SKMEL188 melanoma cells, comparable to that in the rat brain. The respective TPH activities (nmol of Trp hydroxylated/mg protein/h) were 3.03 ± 0.47 ($n = 3$) and 2.39 ± 0.12 ($n = 6$) for rat brain and human melanoma homogenates, respectively.

Using reverse-phase HPLC (RP-HPLC) with fluorimetric detection we conclusively established the presence of AANAT and HIOMT activities in melanoma cells (Figs. 1 and 2). Thus, after the addition of acetyl CoA, cell extracts transformed Ser to NAS and tryptamine to *N*-acetyltryptamine. In addition, melanoma extracts subjected to RP-HPLC showed fluorescence at the RTs of Ser standard, indicating intracellular (endogenous) production of this amine. Fig. 2 shows transformation of NAS to Mel.

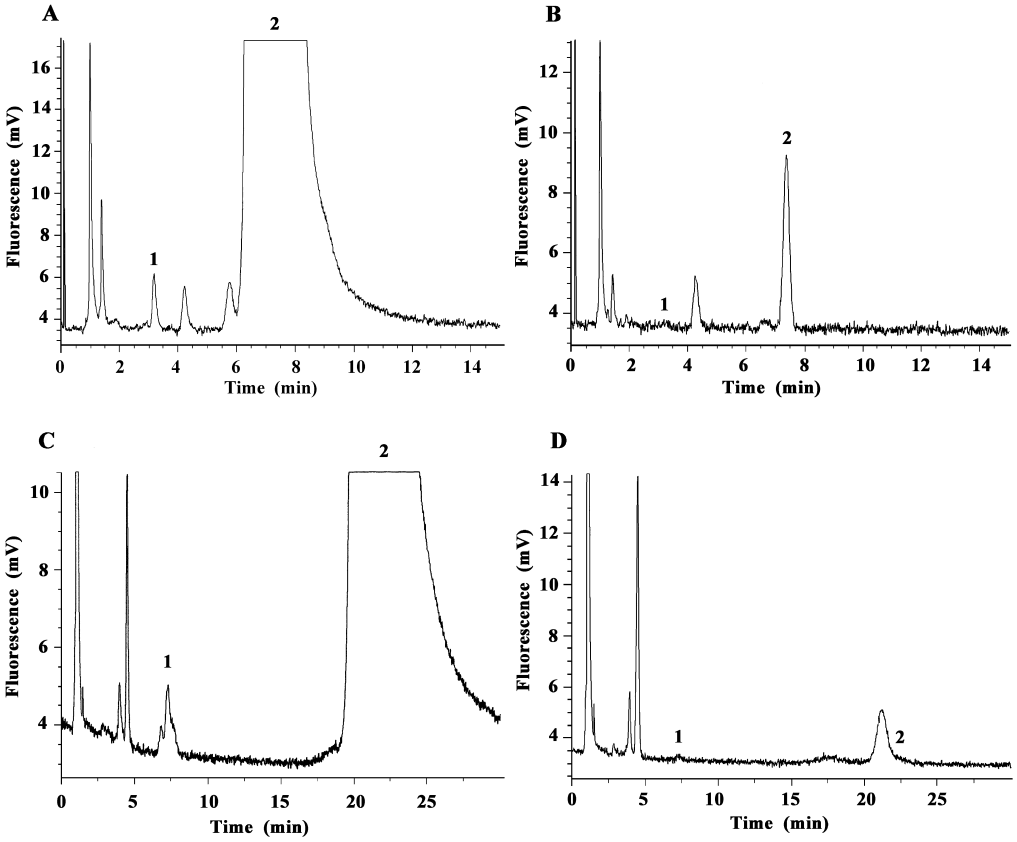

Fig. 1. HPLC chromatogram obtained from reaction mixture in which melanoma cells were the enzyme source: experimental incubation with acetyl CoA and Ser (A) or tryptamine (C) and corresponding control extracts without amine substrate (B and D). The numbers indicate the elution positions of standards. 1 – NAS (A and B) or *N*-acetyltryptamine (C); 2 – Ser (A and B) or tryptamine (C).

3.2. TPH and HIOMT genes expression

Using human-specific primers located at exons 7 and 10 for TPH and exons 4 and 8 for HIOMT we performed RT-PCR on RNA from melanoma cells and detected species of 380 bp for TPH and a 171 bp fragment of HIOMT (Fig. 3). The amplified fragments were sequenced and showed 100% nucleotide sequence homology with the corresponding genes. The PCR fragment of HIOMT corresponded to mRNA lacking exons 6 and 7. This isoform was previously detected by Rodriguez et al. [13] who demonstrated that HIOMT mRNA is produced and alternatively spliced in the pineal gland and also in retina. However, the activity of this HIOMT isoform is still unresolved. The sixth exon of the human HIOMT gene corresponds to the line-1 repetitive element, and this sequence is not found in chicken or cow HIOMT genes. Thus it is assumed that this exon is a recent evolutionary development and is not critical for HIOMT activity [13].

The immunocytochemistry was consistent with gene studies showing in melanoma cells the presence of TPH immunoreactivity and Ser-like immunoreactivity in melanoma cells (not shown).

3.3. LC/MS analysis

Extract ion chromatography analysis at specific masses

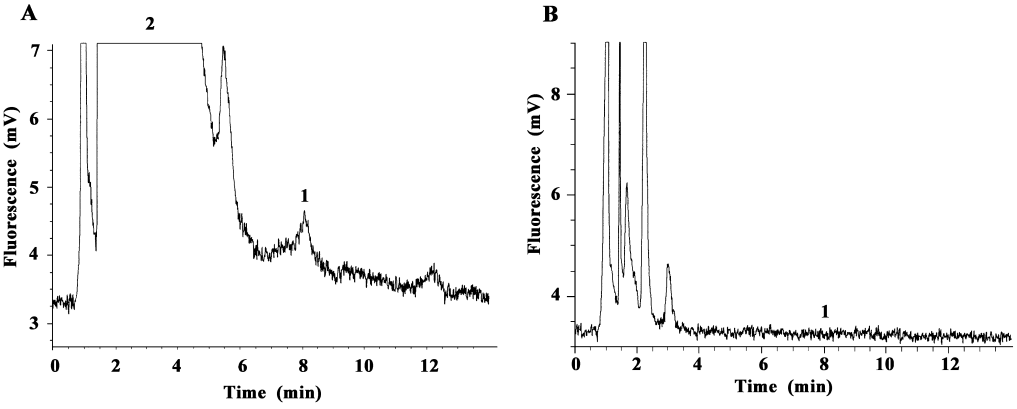

Fig. 2. HPLC chromatogram showing transformation of NAS to Mel in melanoma cell extracts. Experimental incubation with NAS (A) and corresponding control incubation without NAS (B). The numbers indicate the elution positions of standards: 1 – Mel or 2 – NAS.

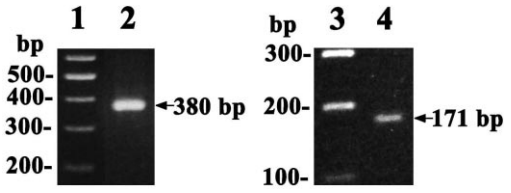

Fig. 3. RT-PCR detection of TPH (lane 2) and HIOMT (lane 4) mRNAs in melanoma cells. Lanes 1 and 3: DNA ladder.

showed adduct ions $(M+H)^+$ at *m/z* 205 with RT 16 min; 191 with RT 17. 1; 192 with RT 22.6; and 233 with RT 23.5 that were identified as Trp, 5MTT, 5MTOL and Mel, because they corresponded to the $(M+H)^+$ of the respective standards with almost identical RT (calculated masses were 204, 190, 191 and 232 Da, respectively) (Fig. 4). The appearance of minor peaks with RT 4.7, 6.4 and 13.3 min at *m/z* 177, 221 and 219, respectively, indicated the presence of Ser, 5-OHTrp and NAS (calculated masses were 176, 220 and 218 Da). The structure of the adduct ions $(M+H)^+$ at *m/z* 221 with RT 7.7 min and 219 with RT 5.6 and 7.7 min and 219 with RT 9 min is unknown.

4. Discussion

We are providing here the first evidence for the existence of a novel melatoninergic system expressed in melanoma cells. This is documented by expression of the TPH gene and its enzymatic activity, detection of AANAT and HIOMT activities with expression of the HIOMT gene. The actual detection of Ser, 5-OHTrp, NAS and Mel in extracts of melanoma cells by LC/MS demonstrates that this melatoninergic system is operating in cultured cells in vitro. Identification of 5MTT and 5MTOL indicates metabolism of Mel to these compounds, following a pathway similar to that described in retina and in frog skin [14].

Ser has multiple functions acting as neurotransmitter, regulator of vascular tone, immunomodulator and as growth factor [2,15]. In the skin, some of the local activities of Ser include those of being pro-edema, vasodilatory, proinflammatory and/or pruritogenic agent [5]. Mel, in turn, can also act as a hormone, neurotransmitter, cytokine, biological modifier and immunomodulator [3]. Thus, the uncovering of endogenous Ser synthesis and its transformation to Mel underlines a putative important role of this pathway in melanocyte physiology and pathology.

One possible melanocytic role for endogenous Mel could be as an intracrine regulator of melanogenesis, or as a scavenger of free radicals produced during melanogenesis, since Mel and related compounds can act as free radical scavengers [16]. Furthermore, in at least some rodent melanomas, Mel has been shown to inhibit melanogenesis (reviewed in [5]). Another possible role for the currently reported serotonergic and melatoninergic systems could be modification of tumor microenvironment by intermediates of the pathway acting in auto- or paracrine fashions. Thus, the present findings uncover a range of new possibilities in pigment and melanoma biology that can be tested in further studies on the role of endogenously produced Ser, Mel and their metabolites.

In conclusion, we provide the first evidence for the expression of novel Ser and Mel biosynthetic pathways in melanoma cells, together with documentation of their possible local degradation.

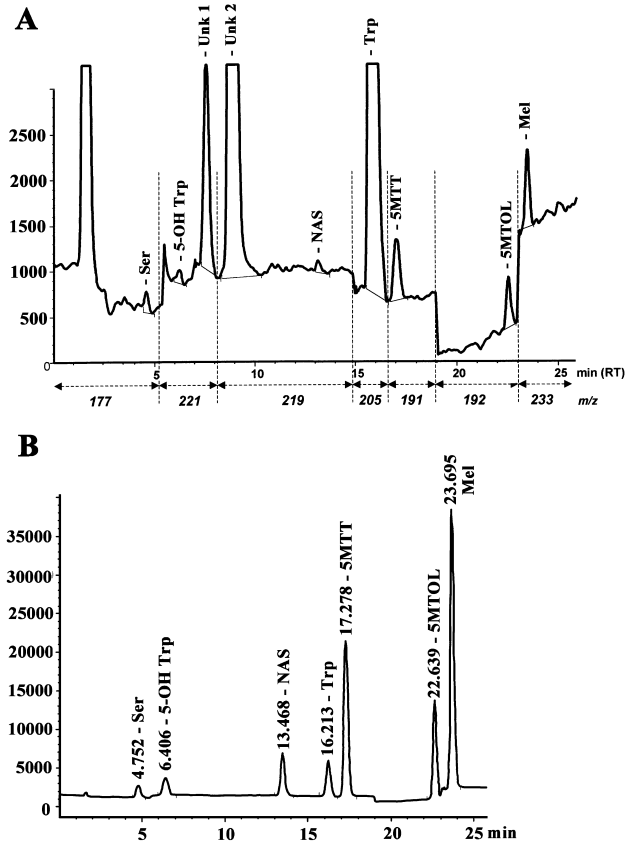

Fig. 4. LC/MS analysis of melanoma cell extracts. A: SIM shows in melanoma extract adduct ions $(M+H)^+$ with *m/z* and RT corresponding to Ser, 5-OHTrp, NAS, Trp, 5MTT, 5MTOL and Mel. B: LC/MS spectra of corresponding standards.

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