Rapid determination of serum melatonin by ESI–MS–MS with direct sample injection

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Abstract

This paper describes a rapid, simple and sensitive analytical method for the quantitative determination of melatonin in human serum by ESI–MS–MS with direct serum sample injection and on-line extraction. The method uses *N*-acetyltryptamine as the internal standard. It has high specificity and sensitivity for serum melatonin analysis. The internal calibration curve shows a wide linear range from 0.500 to 200 ng/ml with a correlation coefficient, $R^2 > 0.999$. The limit of quantitation is 0.500 ng/ml and the limit of detection is 0.100 ng/ml with 10-µl sample injection. The recoveries of serum melatonin at three levels are approximately 70%. The intra-assay precision ($n = 5$) is between 0.8 and 2.0% and the inter-assay precision ($n = 3$) is between 1.5 and 5.9% over the calibration range. This method has a total analysis time of less than 9 min. It can be used for the measurement of melatonin in human blood. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Melatonin; ESI–MS–MS; On-line sample extraction; Direct sample injection

1. Introduction

Melatonin (Fig. 1) is a hormone secreted from the pineal gland in brain, which plays an important role in the regulation of circadian sleep-wake cycle [1]. The concentration of melatonin in blood is changeable with the circadian rhythm, low around 10 pg/ml in the daytime and high around 25–120 pg/ml at night [2]. Melatonin has been orally administered to help patient with jet lag [3], seasonal depression and anxiety symptom [4]. Animal experiment shows that it can improve the immune system and retard the aging process [5]. Melatonin is also a good radical scavenger, suggesting its possible use in the prevention of some forms of cancers [6,7]. Generally, plasma melatonin levels are maintained at about 25–800 times higher than the endogenous peak values after oral administration (1–10.0 mg) [8,9].

Due to its low concentration and the co-existence of many other endogenous compounds in blood, the determination of melatonin has been an analytical challenge [10]. Current analytical

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methods available for blood melatonin include enzyme immunoassay (EIA) [11], radioimmunoassay (RIA) [12], GC–MS methods [13,14], HPLC with fluorescence detection [15,16], and HPLC with electrochemical detection [17], etc. Although EIA and RIA were very sensitive for the determination of melatonin in blood, some anticoagulants and endogenous interfering compounds may affect their specificity [11,12]. The other chromatography-based methods [13–15,17] require prior sample preparations (e.g. liquid–liquid extraction or solid-phase extraction) to remove serum proteins and interfering compounds, which are usually time-consuming and laborious. In case of GC–MS methods [13,14], derivatization of melatonin is needed prior to the analysis.

The purpose of this work is to develop a rapid, sensitive and specific analytical method for the quantitative determination of serum melatonin by ESI–MS–MS with direct sample injection, which employs an on-line sample extraction procedure.

2. Experimental

2.1. Chemicals and solutions

Melatonin (*N*-acetyl-5-methoxytryptamine) and *N*-acetyltryptamine (internal standard) were obtained from Sigma (St Louis, MO, USA). Ammonium formate, formic acid, HPLC grade water
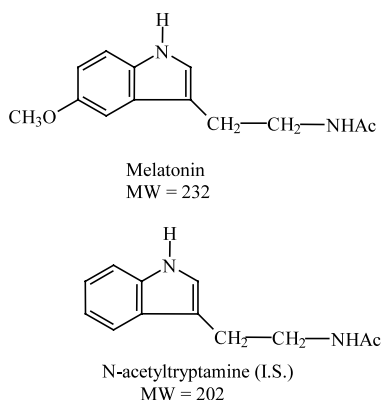

Fig. 1. Molecular structure of melatonin and the internal standard, *N*-acetyltryptamine.

and acetonitrile were from Aldrich (Milwaukee, WI, USA). Human sera (cat. number S7023, lot number 120K8972) was purchased from Sigma and kept at −20 °C.

A stock solution of ammonium formate (50 mM, pH 4.0) was prepared by dissolving appropriate amounts of ammonium formate in a known volume of HPLC grade water. The pH of the solution was adjusted to the desired value with formic acid. Five mM ammonium formate at pH 4.0 was prepared by 1:10 dilution of the stock solution with HPLC grade water.

Stock solutions (1.00 mg/ml) of melatonin and *N*-acetyltryptamine (0.500 mg/ml, internal standard) were prepared by dissolving appropriate amounts of each compound in a known volume of methanol, which were stored at −20 °C when not used.

Working standard solutions of melatonin (8.00, 80.0, 600 and 1000 ng/ml) and *N*-acetyltryptamine (8.00, 80.0, 200 and 600 ng/ml) were prepared by serial dilution of the stock solutions with 5 mM ammonium formate (pH 4.0).

2.2. Blank sera, serum standards and samples

Human sera contained no detectable melatonin were used as the blank sera. Serum standards (0.500, 1.00, 2.00, 5.00, 15.0, 50.0, 100 and 200 ng/ml) were prepared by the serial dilution of the 1000-ng/ml melatonin working standard solution with the blank sera.

Prior to the analysis, 10-μl internal standard solution (*N*-acetyltryptamine, 200 ng/ml in 5 mM ammonium formate at pH 4.0) was added to 200-μl serum standards and serum samples. The mixtures were diluted with 190-μl, 5 mM ammonium formate (pH 4.0). After vortex mixing, these solutions were transferred to autosampler vials for instrument analysis.

2.3. Instrumentation

The instrumentation system used for this work consisted of an HP1100 pump (Hewlett-Packard, Palo Alto, CA, USA), an HP1100 autosampler, a stainless steel in-line filter (0.5-μm pore, 0.23-μl dead volume, Upchurch Scientific, Oak Harbor,
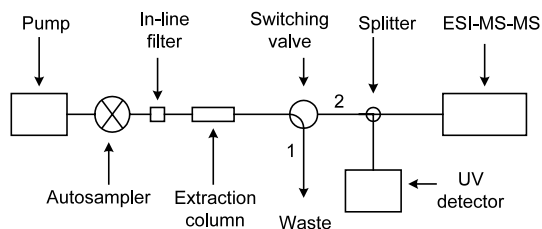
Fig. 2. Block diagram of the instrumentation system.

Table 1
The flow profile of the instrumentation system

Time (min)	A (%)	B (%)	Flow rate (ml/min)
0.0	100	0	1.0
2.0	100	0	1.0
2.1	0	100	0.2
7.5	0	100	0.2
7.6	0	100	1.0
8.0	0	100	1.0
8.1	100	0	1.0
9.0	100	0	1.0

A, 5 mM ammonium formate at pH 4.0. B, 95% acetonitrile + 5% 5 mM ammonium formate at pH 4.0.

WA, USA), an Oasis® HLB extraction cartridge column (2.1 × 20 mm, Waters, Milford, MA, USA), a Rheodyne 7000 two-position 6-port switching valve (Supelco, Bellefonte, PA, USA), a stainless steel splitting tee (1/16 in. × 0.25 mm, Valco, Houston, TX, USA), an HP1100 variable wavelength UV detector, and a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). The fluidic connections of the system is shown in Fig. 2. At the position 1, the extraction buffer from the pump carried the sample from the autosampler to the extraction column where the sample matrix was excluded to the waste and analytes of interest were retained on the column. At the position 2, the elution buffer from the pump eluted the analytes and the internal standard from the extraction column and carried them to the splitter where the flow was diverted to the ESI-MS detector and the UV detector with a split ratio of 1:2. The smaller flow went to the MS detector (ca. 70 μl/min) and the larger one to the UV detector. For all the connections, high-pressure polyether ether ketone (PEEK) tubing (0.0625 in. O.D.) was used. From the splitter to the ESI-MS detector, 0.005 in. I.D. tubing was used, and for the rest of connections, 0.01 in. I.D. tubing was used.

2.4. On-line sample preparation

At the position 1 of the switching valve (Fig. 2), Oasis® extraction column was first equilibrated with the extraction buffer (5 mM ammonium formate at pH 4.0) at a flow rate of 1.0 ml/min. Then, a 10-μl portion of the diluted serum sample was injected by autosampler onto the extraction column. This step excluded the sample matrix to the waste and retained the analytes of interest on the column. After 2-min sample extraction and cleanup, the switching valve was turned to the position 2. At the same time, the extraction buffer was changed to the elution buffer (95% acetonitrile + 5% 5 mM ammonium formate at pH 4.0) with a flow rate of 0.2 ml/min, which initiated the elution of analytes from the extraction column. After 5.5-min elution, the switching valve was turned back to the position 1. The elution buffer was pumped through the column at 1 ml/min for 0.5 min before it was changed back to the extraction buffer. The extraction column was equilibrated with the extraction buffer at 1 ml/min for 1.0 min before the next injection. The complete flow profile was summarized in Table 1 and the total run time per sample was 9 min.

2.5. ESI–MS–MS detection

The Quattro II triple quadrupole mass spectrometer was operated in the positive electrospray ionization mode (ESI+). It was tuned by infusion of a mixture of melatonin and N-acetyltryptamine (1.00 μg/ml each) in 50% acetonitrile + 50% 5 mM ammonium formate (pH 4.0) at a flow rate of 3 μl/min with a syringe pump (Harvard Apparatus, South Natick, MA, USA). The optimzed ionization conditions were: nitrogen sheath and desolvation gas at 10 and 350 l/h, capillary at 3.5 kV, HV lens at 0.5 kV, cone at 25 V, skimmer at 1.5 V, RF lens at 0.2 V, ion source temperature at 70 °C, ion energy at 0.6 V, low- and high-mass resolution at 15, and multiplier at 650 V.

Full scan spectra were acquired in the continuum mode over the mass range of 180–280 u at the scan rate of 500 u/s. Selected ion recording (SIR) data were acquired by sequentially monitoring quasi-molecular ions of the analytes ([melatonin + H]$^+$ at m/z 233 and [N-acetyltryptamine + H]$^+$ at m/z 203) with a span of 0.05 u, a dwell time of 0.5 s, and an inter-scan delay of 0.03 s. Ionization parameters were the same as those described above.

Multiple reaction monitoring (MRM) data were acquired with the following parameters: m/z 233 > 174 for melatonin, m/z 203 > 144 for N-acetyltryptamine, argon collision gas at 1.0 μbar, cone at 25 V, collision energy at 15 eV for each analyte, low- and high-mass resolution at 15 for both quadrupole 1 and 3, dwell time of 0.5 s, and inter-scan delay of 0.03 s. The ionization parameters used for MRM were the same as those for SIR.

2.6. Quantitation

Data acqusition was done by MICROMASS MASSLYNX software (Version 3.4). The peak area ratios of melatonin to the internal standard (Y-axis values) were plotted against the melatonin concentrations (X-axis values) for the regression equation. The serum melatonin concentration of the unknown sample was determined by the regression equation after obtaining the peak area ratio of the unknown to the internal standard from the mass chromatograms.

2.7. Recovery

The three-concentration-level (2.00, 20.0 and 150 ng/ml) serum samples used in the recovery studies were prepared by adding 50-μl internal standard (N-acetyltryptamine, at 8.00, 80.0 and 600 ng/ml in 5 mM ammonium formate at pH 4.0), 50-μl melatonin (at 8.00, 80.0 and 600 ng/ml in 5 mM ammonium formate at pH 4.0), and 100-μl of 5 mM ammonium formate (pH 4.0) to 200-μl blank serum. The control samples were prepared in 5 mM ammonium formate (pH 4.0) at the same concentration levels. The recoveries of melatonin and N-acetyltryptamine in human sera were determined by comparing the mean peak areas of serum samples to the mean peak areas of the control samples.

3. Results and discussions

3.1. On-line sample preparation

Sample preparation is the bottleneck of most of drug analyses in biological fluids. Direct sample injection with on-line sample preparation has been developed to simplify and expedite the analytical procedure for drug analyses. In this work, Waters Oasis®HLB extraction cartridge was used as an on-line column for the extraction of serum melatonin and N-acetyltryptamine (internal standard). According to the manufacturer, the packing material of the extraction column is a macroporous copolymer [poly(divinylbenzene-co-N-vinylpyrrolidone)] and HLB is an acronym for hydrophillic lipophillic balance which describes the dual retention capability of the packing material to retain polar and nonpolar compounds. The water wettable polymeric sorbent of Oasis®HLB allows the exclusion of proteins and other interfering matrix constituents, and the retention of acids, bases and neutral compounds with high and reproducible recoveries [18].

By the experiments shown in Fig. 3, it was found that serum proteins and other interfering matrix constituents were completely eluted in less than 1 min after sample injection using 5 mM ammonium formate (pH 4.0) at the flow rate of 1.0 ml/min as the extraction buffer (Fig. 3A). Meanwhile, melatonin, N-acetyltryptamine and other endogenous compounds were retained on the column even after 9-min washing with the extraction buffer (Fig. 3B). Therefore, 2-min after sample injection was chosen as the switching time when the switching valve was turned from the position 1 to 2 (Fig. 2). The elution of melatonin and N-acetyltryptamine as well as other endogenous compounds took place after the extraction buffer was changed to the elution buffer (95% CH$_3$CN + 5% 5 mM ammonium formate at pH 4.0) at 2 min of the sample injection. Fig. 3C and D showed that these compounds reached the UV

detector after 5.8 min of sample injection. To avoid the contamination of ESI–MS–MS detector, only UV detector was used to monitor the elution profiles of serum samples in these experiments.

It was also known from the experiments that low percentage of CH_3CN in the elution buffer would increase the retention time of the analytes and longer retention could result in further dilution of the analytes on the extraction column. In this work, 95% CH_3CN was used for the rapid elution of the analytes, and 5% 5 mM ammonium formate (pH 4.0) was used to suppress the formation of sodium adducts and to increase the ionization efficiency of the analytes.
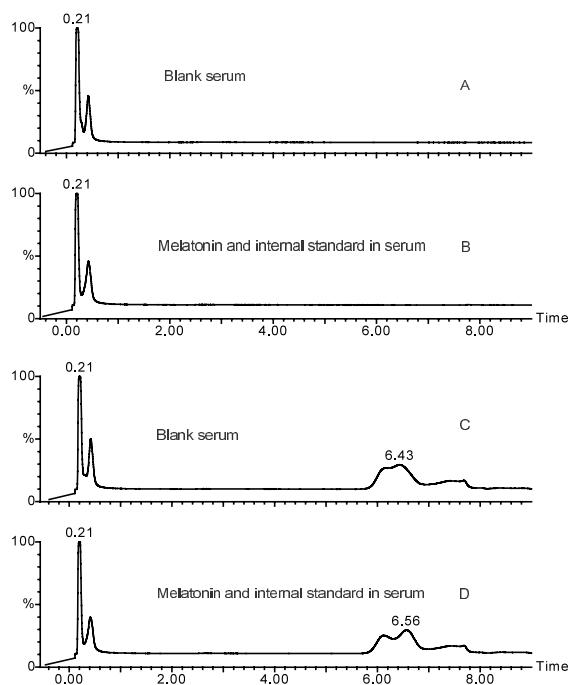

Fig. 3. UV chromatograms of blank human serum (A, C) and human serum spiked with 200 ng/ml melatonin and N-acetyltryptamine (B, D). For A and B, 5 mM ammonium formate at pH 4.0 was the carrier fluid; for C and D the carrier fluids was the same as those in Table 1. Instrumentation, Fig. 2 at the position 2 without ESI–MS–MS detector; and UV detection wavelength, 254 nm.
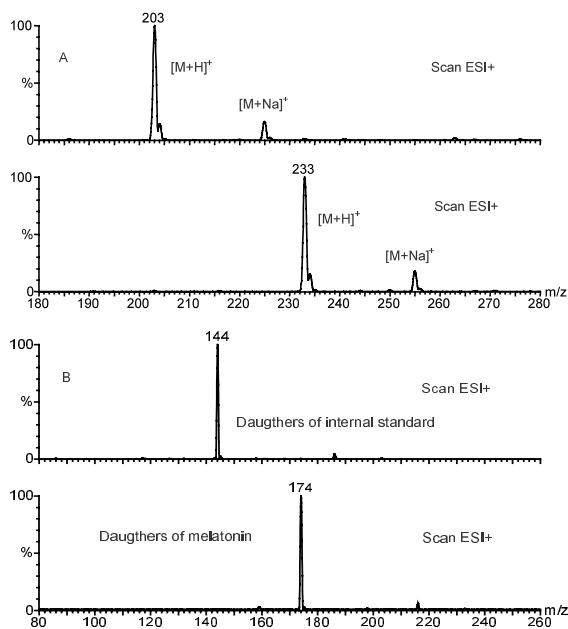

Fig. 4. Positive-ion scan spectra of melatonin and N-acetyltryptamine (internal standard). (A) the parent ions; (B) the daughter ions. Instrumentation system: Fig. 2 without the UV detector. Detection conditions: the same as those described in Section 2.5.

3.2. ESI–MS–MS detection

Melatonin and N-acetyltryptamine are more readily obtaining a proton than losing one in the acidic electrospray ionization conditions; therefore, the positive electrospray ionization mode has been the choice of detection. Full scan spectra over the mass range of 180–280 u revealed that melatonin produced a quasi-molecular ion $[M + H]^+$ at m/z 233 and N-acetyltryptamine at m/z 203 (Fig. 4A). By selecting these quasi-molecular ions as parent ions, daughter spectra could be obtained. The prominent daughter ions over the range of 80–260 u were m/z 174 for melatonin and m/z 144 for N-acetyltryptamine (Fig. 4B), which were produced by colliding with argon gas in the quadrupole 2 of the mass spectrometer and losing a neutral fragment of 59 u (CH_3CONH_2) from the parent ions.

In this work, since no separation column was used, many endogenous interfering compounds co-eluted with melatonin and the internal stan-

dard from the extraction column. By comparing mass chromatograms of 'single ion recording,
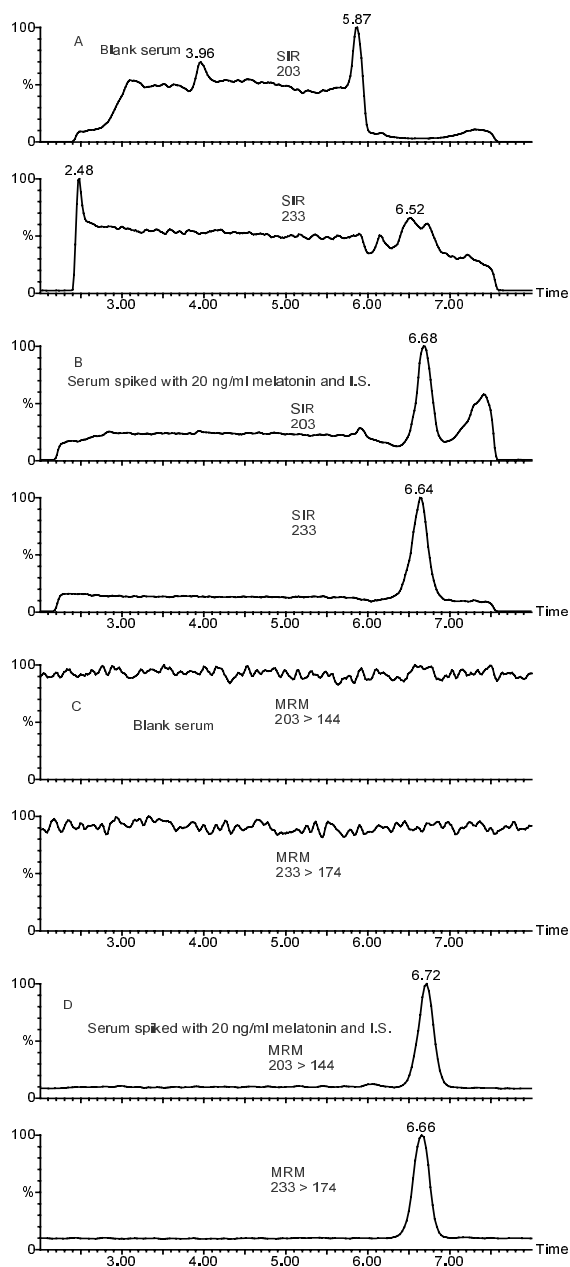

Fig. 5. SIR and MRM chromatograms of melatonin and *N*-acetyltryptamine in human sera. SIR mode: m/z 233 for melatonin and m/z 203 for *N*-acetyltryptamine. MRM mode: m/z 233 > 174 for melatonin and m/z 203 > 144 for *N*-acetyl-trypamine. Experiment conditions, the same as those in Fig. 4.

SIR' (Fig. 5A and B) to those of MRM (Fig. 5C and D), it was clearly evident that MRM mode experienced no interference from the sample matrix. Therefore, it was chosen as the detection mode for melatonin quantitation.

The settings of low- and high-mass resolution for quadrupole 1 and 3 could affect both the flux of ions and the noise levels. To obtain the highest signal-to-noise ratio, a setting of 15 for both low- and high-mass resolution was found to be the optimum for this work.

Although a relatively long scan time (dwell time) could decrease the sensitivity a little, it remarkably improved the signal-to-noise ratio. A 0.5-s scan time was used for the detection of melatonin and the internal standard.

It was also noticed that a low collision gas pressure in the quadrupole 2 could decrease the noise levels in the MRM mode. Therefore, an argon gas pressure of 1.0 μbar was chosen for this work.

3.3. Flow rate

The flow rate of the elution buffer affected the detection sensitivity of the MS in rather a remarkable way. It was found that the sensitivity increased as flow rate decreased. As shown in Fig. 6, the peak areas and heights of melatonin and *N*-acetyltryptamine became larger as the flow rate decreased from 1 to 0.15 ml/min. A reasonable explanation is that ESI-MS is a concentration-sensitive detector. When the amounts of analytes were fixed, the fluidic volumes delivered per unit time were higher at higher flow rates, and the actual concentrations of the analytes in the MS were lower than those at lower flow rates.

It was also noticed that when the flow rate got too small, the elution time and the peak width could become longer and broader. Therefore, a flow rate of 0.2 ml/min was chosen for the subsequent studies, which gave an adequate detection sensitivity and elution time. At this flow rate, the actual fluid flowed into the MS was about 70 μl/min due to the post-column splitting at a ratio of 1:2.
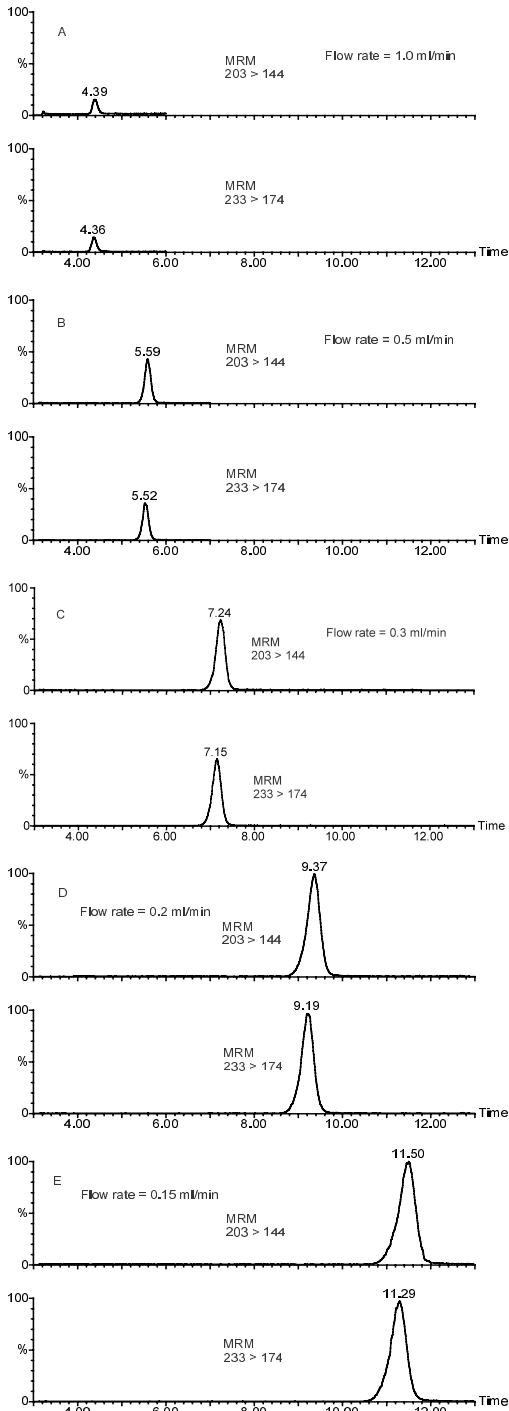
Fig. 6. Effect of flow rate on signal responses of melatonin and *N*-acetyltrypamine. Experimental conditions were the same as those described in Section 2.4 except the elution buffer were 47.5% acetonitrile + 52.5% 5 mM ammonium formate (pH 4.0) and the switching time were 3-min after sample injection.
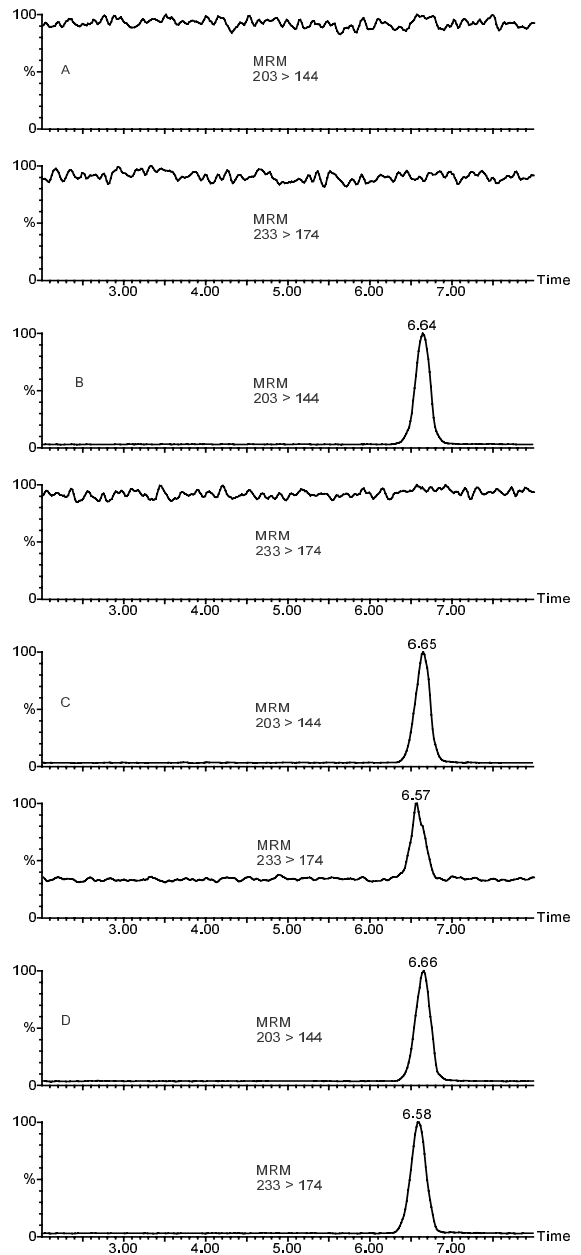
Fig. 7. Mass chromatograms of MRM detection mode. (A) Blank human serum; (B) blank human serum spiked with 20 ng/ml *N*-acetyltrypamine (internal standard); (C) blank human serum spiked with 20 ng/ml internal standard and 1 ng/ml melatonin; and (D) blank human serum spiked with 20 ng/ml internal standard and 20 ng/ml melatonin. Experiment conditions: the same as those in Fig. 4. Melatonin, m/z 233 > 174; internal standard, m/z 203 > 144.

Table 2
Recoveries of melatonin and N-acetyltryptamine (I.S.) from human sera

Compounds[a]	2.00 ng/ml		20.0 ng/ml		150 ng/ml	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Melatonin	69.1	5.1	68.9	3.6	70.5	1.9
I.S.	59.1	1.7	53.2	4.5	57.7	0.67

Recovery = Mean peak area of serum samples ($n = 3$)/Mean peak area of control samples ($n = 3$) × 100%.
  [a] Injection volume = 10 μl.

3.4. Sodium adducts

Melatonin and N-acetyltryptamine could easily form adducts with sodium ion in the positive electrospray ionization process. The formation of sodium adducts $[M + Na]^+$ competed with the formation of quasi-molecular ions $[M + H]^+$ and affected the detection sensitivity. Hence, it should be suppressed. The experiments indicated that ammonium formate was more effective in suppressing the formation of sodium adducts than formic acid; whereas formic acid might cause a low ionization efficiency of the analytes as trifluoroacetic acid (TFA) did in the previous study [19]. For the subsequent studies, 5% 5 mM ammonium formate (pH 4.0) was used with 95% CH_3CN as the elution buffer for achieving high detection sensitivity.

3.5. Analytical performance

The MRM mode was used for quantitation. Its specificity for detecting melatonin and N-acetyltryptamine was illustrated by the representative mass chromatograms in Fig. 7, which showed no sign of interference from the co-eluting endogenous compounds in the serum matrix.

The recovery studies of melatonin and N-acetyltryptamine were conducted at three-concentration levels (2.00, 20.0 and 150 ng/ml) and the results were summarized in Table 2. The absolute recoveries of melatonin and N-acetyltryptamine were 68.9–70.5 and 53.2–59.1%, respectively. The recovery data showed good consistency at all levels. It is noted that recoveries of analytes decreased gradually with a sample injection volume greater than 50 μl (data not shown), which were

probably due to the loading capacity of the extraction column at given concentration levels. In this work, an injection volume of 10 μl was used for quantitation.

The method developed has excellent intra- and inter-assay precision for melatonin. As shown in Table 3, The relative standard deviations of melatonin at four-concentrations for the within-day (intra-assay) study ranged 0.8–2.0% ($n = 5$) and the day-to-day (inter-assay) study ranged 1.5–5.9% ($n = 3$).

A good linear relationship was found between the peak-area ratios of melatonin to N-acetyltryptamine (the internal standard) versus the concentrations of melatonin ranging 0.500–200 ng/ml. Linear regression analysis indicated that the correlation coefficient (r^2) was greater than 0.999, and the regression equation was $Y = 0.129 X + 0.001$ with Y as the peak area ratio and X as the concentration of melatonin.

The measured serum melatonin concentrations were found to be in good agreement with the actual concentrations of melatonin prepared by spiking melatonin in human blank sera. The accuracy bias shown in Table 4 ranged from -0.80 to -5.9% at the four concentrations evaluated.

Table 3
Intra- and inter-assay precision of serum melatonin

Melatonin (ng/ml)	Intra-assay (%CV, $n = 5$)	Inter-assay (%CV, $n = 3$)
1.00	2.0	5.9
5.00	0.80	1.8
25.0	1.8	1.5
150	1.7	2.5

Table 4
Accuracy of the serum melatonin measurement

Melatonin added (ng/ml)	Melatonin measured (ng/ml)	Accuracy bias (%)[a]	%CV ($n = 5$)
1.00	0.941	−5.9	2.0
5.00	4.95	−1.0	0.80
25.0	24.8	−0.80	1.8
150	146	−2.7	1.7

[a] Accuracy bias (%) = (measured value − nominal value)/nominal value × 100.

The limit of detection defined as the signal-to-noise ratio of 3 was 0.100 ng/ml for serum melatonin ($n = 3$) and the limit of quantitation defined as the lowest detectable analyte concentration which has an accuracy bias of 18% was 0.500 ng/ml for serum melatonin ($n = 3$).

In comparison with the previously reported atomspheric pressure chemical ionization (APCI)-MS-MS method [20], it is clearly evident that our ESI–MS–MS method for serum melatonin has lower limit of detection and wider linear dynamic range, are capable of handling larger amount of sample and higher flow rates.

3.6. Applicability to pharmacokinetic studies

According to Dollins et al. [9], melatonin doses at 1 mg or greater were needed to generate peak serum melatonin levels that exceeded the normal range of nocturnal melatonin levels in untreated people, which corresponded to peak serum melatonin levels 0.500 ng/ml or higher.

The method developed has a limit of quantitation of 0.500 ng/ml with 10-μl sample injection, which is suitable for the pharmacokinetic study of melatonin at doses greater than 1 mg. It should also be pointed out that the extraction cartridge used in this work has concentration capability, which allows us to injection sample volumes up to 50 μl without sacrificing the analyte recoveries. Therefore, if lower limits of quantitation are desirable, larger sample volumes can be used. Although the method developed has not yet been tested with clinical samples, its applicability to serum melatonin has been demonstrated.

4. Conclusions

A rapid, simple and sensitive analytical method for the quantitative determination of melatonin in human serum has been developed and validated. It employs on-line sample preparation and ESI–MS–MS detection. Serum samples can be directly injected into the system and be analyzed within 9 min. The method has wide linear dynamic range, low limit of quantitation, high specificity and sensitivity, as well as excellent reproducibility and accuracy. It may be used for the pharmacokinetic study of melatonin in human sera.

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