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Determination of melatonin in biological fluids in the presence of the melatonin agonist S 20098: comparison of immunological techniques and GC-MS methods ☆

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Abstract

Immunoassays were investigated for the determination of melatonin in biological samples in the presence of a naphthalenic structural analogue S 20098, which is currently under development as a melatonin agonist. The lack of specificity of commercially available antibodies in the presence of closely related molecules led us to develop an LC-RIA procedure with a quantification limit set at 15 pg ml^{-1}. Because this technique was not sensitive enough and difficult to use on a routine basis, a more sensitive GC-MS technique was developed. This method involved automated solid-phase extraction (plasma) or liquid–liquid extraction (saliva), derivatization of the indolic moiety and GC separation with an automated switching device before MS detection. The method was validated over the range 1–100 pg ml^{-1}, with a quantification limit set at 1 pg ml^{-1} in human plasma and saliva. Intra-assay and inter-assay precision and accuracy were within 16% for all concentrations investigated and each biological matrix. The stability of melatonin in plasma and saliva under various storage conditions was also determined. The specificity of the assay for the analysis of melatonin in the presence of S 20098 and its metabolises was demonstrated. The method was subsequently applied for the determination of endogenous melatonin concentrations in plasma and saliva samples from clinical studies performed with S 20098 to provide pharmacodynamic data.

Keywords: Radioimmunoassay; Capillary gas chromatography; Mass spectrometry; Melatonin; Plasma; Saliva

1. Introduction

The hormone melatonin (*N*-acetyl-5-methoxytryptamine) plays an important role in the circadian regulation of various physiological and neuroendocrine processes. The synthesis of

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melatonin and its secretion at night from the mammalian pineal gland is controlled by a circadian clock within the hypothalamic suprachiasmatic nucleus. The development of a new melatonin receptor agonist [1] S 20098 (Fig. 1, 1) required the specific measurement of melatonin in biological samples at very low concentrations in order to accurately determine the circadian rhythm of this endogenous hormone before and after administration of S 20098. Several analytical techniques have been previously reported for the analysis of melatonin in biological fluids, such as urine, plasma, saliva or pineal gland. High-performance liquid chromatographic techniques with either electrochemical [2–5] or fluorimetric detection [6–9] have been described. Although very simple to use, these methods are not sensitive enough for the measurement of melatonin in plasma and saliva, since the limit of detection was set around 50 pg ml^{-1} for plasma (1 ml), and 3 pg ml^{-1} for the pineal gland [9].

Immunoassay techniques have been extensively employed for the determination of melatonin in biological fluid due to their simplicity of use and high sensitivities. Two different routes have been followed to raise antibodies against melatonin.

The first antibodies described in the literature were obtained after immunization of immunogens obtained from the coupling of a melatonin hapten to a protein by Mannick condensation with formaldehyde [10,11]. Using these immunogens, the antibodies obtained in sheep or rabbit can be used with [^{125}I]2-iodomelatonin as a radioligand, giving high sensitivities, down to 2 pg ml^{-1} in saliva and 5 pg ml^{-1} in plasma after extraction [12,13].

Nonetheless, only limited specificity is obtained with these antibodies and many indolic substituted analogues, such as 6-hydroxymelatonin, exhibit significant cross-reactivities (5%) [13].

A second route has been also described to raise antibodies against melatonin, using immunogens obtained from the coupling of a carboxylated melatonin derivative (5-methoxy-*N*-acetyl-tryptophan) to BSA or thyroglobulin [14,15]. The antibodies obtained gave a specificity which was directed towards both 5-methoxyindole and the acetamide group. Substituted melatonin analogues such as 6-hydroxymelatonin have very low cross-reactivities (0.02 to 0.6% depending on the proteins used as immunogens [16]). These antibodies could therefore be used directly in saliva or in plasma samples without the need for extraction, however, the sensitivity is only 10 pg ml^{-1} using 500 μl plasma and [3_H]*O*-methylmelatonin as a radioligand [13]. To increase sensitivity, many authors have developed sample preparation methods to concentrate the analyte [18,19]. In spite of

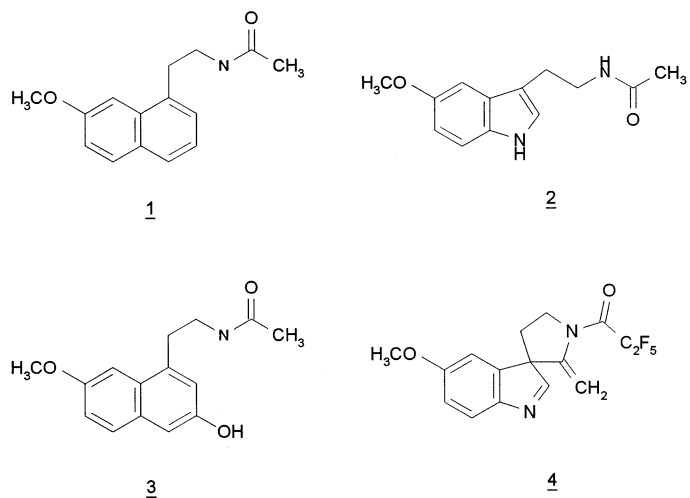

Fig. 1. Chemical structures: 1, melatonin agonist S 20098; 2, melatonin; 3, 3-hydroxy metabolite of S 20098; 4, spirocyclic fluoroderivative of melatonin.

these methods, the sensitivity is always limited by the use of the tritiated radioligand, since 2-iodomelatonin could not be used with these antibodies. Some enzyme immunoassays have also been developed with these antibodies, but with lower sensitivities compared with radioimmunoassays [16,17,20].

The specificities of both types of antibodies have been tested for the analysis of melatonin in the presence of S 20098. Due to the significant cross-reactivities of this compound with both antibodies an LC-RIA method was developed: this method included the chromatographic separation of S 20098 and its metabolites from melatonin derivatives, followed by RIA detection of melatonin after fraction collection. The limit of quantification was only set at 15 pg ml^{-1}, with a calibration range of 5–200 pg ml^{-1}. Another technique was developed to increase the sensitivity of the method whilst retaining specificity: this technique involved gas chromatography and mass spectrometry. The latter technique has also been described for the determination of melatonin. The technique involves acylation of melatonin with pentafluoropropionic anhydride (PFPA) to give a spirocyclic derivative [21,22], followed by gas chromatographic separation and mass spectrometric detection in the electron impact mode [23,24], or most commonly in the negative ion chemical ionization mode [25–29]. These methods have been described for melatonin determination in plasma, serum [23,25,27–29] or in saliva [26,30,31] and in other tissues such as the pineal gland or the retina with liquid–liquid extraction commonly employed for sample preparation. Interestingly, the use of a switching device during gas chromatographic analysis [23,24,32] has allowed melatonin levels of down to 1 pg ml^{-1} to be detected from the inclusion of a supplementary purification step. GC-MS methods have been used to a lesser extent in comparison with immunological techniques, due to their lack of automation for routine analysis. The present work describes the development and validation of a fully automated GC-MS technique for melatonin quantification in the presence of S 20098 in human plasma and saliva samples. The method involves the automated solid-phase extraction of plasma samples, or liquid–liquid extraction of saliva samples, followed by GC-MS separation with an automated switching device incorporated as an additional purification step. A complete validation of this technique is described for both of these biological matrices. This method was then applied for the determination of melatonin concentrations in biological samples from clinical studies following administration of the melatonin agonist S 20098.

2. Materials and methods

2.1. Chemicals and Reagents

Melatonin was purchased from Fluka (Buchs, Switzerland), the internal standard (i.e. deuterium labelled melatonin or *N*-(2-(5-methoxy-1*H*-indol-3-yl)[$^2H_4$]ethyl)[$^2H_3$]acetamide) was synthesized by Technologie Servier (Orleans, France) and both radioligands [^{125}I]-2-iodomelatonin (74 TBq $mmol^{-1}$) and [^{3}H]*O*-methylmelatonin (3.15 TBq $mmol^{-1}$) were purchased from Amersham (Les Ulis, France).

The two different antibodies used were purchased from Stockgrand (Guildford, UK) reference 19540/16876 and reference HP/S 704-8483. Tricine buffer (0.1 M) containing sodium chloride 0.9% and gelatine 0.2% was adjusted with 1 M NaOH to pH 8 or 5.5 depending on the assay.

Donkey rabbit antiserum was purchased from Cis Bio International (Gif-sur-Yvette, France). Charcoal Norit A was purchased from Serva (Paris, France) and Dextran T-70 from Pharmacia (St-Quentin-en-Yvelines, France). The scintillation reagent Ultima Gold was purchased from Packard (Rungis, France).

Dichloromethane, diethylether, ethanol, ethylacetate, *n*-hexane, acetonitrile and methanol were of analytical grade. Sodium hydroxide (0.2 M) and potassium chloride (0.2 M) were of reagent grade and used with distilled water for the preparation of pH 13 buffer (respectively 66:25:9, v/v/v). Pentafluoropropionic anhydride (PFPA) was supplied in 1 ml sealed ampoules from Aldrich (St-Quentin-Fallavier, France). Blank human plasma samples were obtained from the 'Centre

Departemental de Transfusion Sanguine' (Orleans, France) and blank human saliva samples from healthy volunteers. Plasma and saliva samples were collected in the beginning of the afternoon to obtain control samples with very low concentrations of endogenous melatonin. The blank plasma and saliva samples were routinely checked before use for the preparation of standards and quality control samples.

Solid-phase extraction cartridges C8 Amprep (1 ml) were purchased from Amersham.

2.2. Radioimmunoassays

The RIA procedures have been described previously [11,14]. They are briefly outlined below.

2.2.1. Antisera 19540/16876

A solid-phase extraction of plasma samples (1 ml) on C8 Amprep cartridges (see Section 2.3 below) was performed with an ASPEC system. After evaporation, the extract was dissolved in Tricine buffer pH 8 (0.5 ml) before incubation of the samples in triplicate (3×100 μl) with the corresponding rabbit antiserum (1:30 000 dilution in Tricine buffer pH 8) and [^{125}I]2-iodo melatonin (100 μl, 10 000 cpm) for 3 h at 4°C. The mixture was incubated for 15 min at 4°C after addition of Donkey rabbit antiserum (1 ml) and centrifuged (5000 rpm) for 20 min at 4°C. The supernatant was then discarded and the radioactivity in the pellets was counted (Cobra system, Packard).

2.2.2. Antisera HP/S/704-8483

The same solid-phase extraction procedure for plasma samples (1 ml) was performed as described above. After evaporation, the extract was dissolved in Tricine buffer pH 5.5 (300 μl) and incubated with appropriate sheep antiserum (100 μl, 1:5 000 dilution) and [^{3}H]*O*-methylmelatonin as radioligand (100 μl, 15 000 dpm) and incubated for 3 h at 4°C. Activated coated charcoal (6 g l^{-1}) containing dextran (0.5 g l^{-1}) was then added (1 ml) and the samples were centrifuged (4000 rpm) for 15 min and at 4°C. The supernatant (1 ml) was then removed for counting with liquid scintillation fluid (4 ml) in a β-counter (Packard).

2.2.3. LC-RIA procedure

The first step was the solid-phase extraction of human plasma samples (1 ml) on C8 Amprep cartridges as described below for the GC-MS procedure. Following evaporation, the extracts were dissolved in distilled water (250 μl) before chromatographic separation on a C8 Ultrasphere analytical column (150×4.6 mm, 5 μm) from Beckman (Gagny, France). The mobile phase consisted of a concentration gradient of water (A) and acetonitrile (B) set up to flow at a rate of 1 ml min^{-1} as follows: $t = 0$ until 10 min A:B, 75:25 (v/v); $t = 10$ min until 14 min A:B, 10:90 (v/v), $t = 14$ min until $t = 19$ min A:B, 75:25 (v/v). 100 μl of sample were injected and the fractions were collected between 4.8 and 7.8 min at 8°C in the absence of light. RIA detection was performed as described above for the second RIA procedure (antiserum HP/S/704-8483) after evaporation of the chromatographic extracts and solubilization in Tricine buffer pH 5.5 (400 μl). The incubation time was 12 h, instead of 3 h, at 4°C

Chromatographic separations were performed with an AS 3000 automated injector, a SP8800 gradient pump from TSP (Les Ulis, France) and a fraction collector from Pharmacia.

2.3. GC-MS procedure

2.3.1. Extraction of the samples

The same solid-phase extraction procedure was used for immunoassays and chromatographic techniques, with the addition of an internal standard for the GC-MS method only.

Melatonin was extracted from plasma by solid-phase extraction with C8 Amprep cartridges (1 ml) on an ASPEC XL system from Gilson (Villiers-le-Bel, France). Each plasma sample (1 ml) was spiked with 10 μl of the internal standard solution and a pH 13 buffer solution (0.2 ml) and loaded onto cartridges previously washed with methanol (2 ml) and water (2 ml). The cartridges were then washed with water (2 ml) and dried with *n*-hexane (2 ml). Melatonin (and the internal standard when appropriate) were then eluted with diethylether–dichloromethane (60:40, v/v, 3 ml). The extract was evaporated to dryness before derivatization (GC-MS procedure) or immuno-

analysis. Extraction from saliva was performed by liquid–liquid extraction. Saliva samples (1 ml) spiked with 10 μl of the internal standard solution were washed with 5 ml of *n*-hexane and the organic layer was discarded. The aqueous phase was then made alkali with 250 μl of 1 M NaOH and shaken with dichloromethane (5 ml). After centrifugation, the organic layer was sampled and evaporated to dryness under nitrogen.

2.3.2. Derivatization

The dry residues obtained from plasma or saliva samples were taken up in dry ethyl acetate (0.2 ml) and PFPA (25 μl) was added. After heating at 60°C for 15 min, the contents were evaporated to dryness. Ethyl acetate (25 μl) was added to the residues and 2-μl samples were injected for GC-MS.

2.3.3. Chromatographic conditions

A 5989A Hewlett-Packard mass spectrometer coupled to an HP 5890 gas chromatograph was used. The GC oven was equipped with a capillary column switching system (Multi Dimensional System 2000 SGE) and a liquid carbon dioxide trap. Chromatographic columns incorporated a retention gap of deactivated fused silica with methyl silicone (about 1 m × 0.53 mm): an initial analytical column of fused silica (12.5 m × 0.32 mm) with a methyl silicone bonded phase (BPS, SGE) and a second analytical column (25 m × 0.22 mm) of methyl silicone (BPX5, SGE), which was directly connected to the ion source. Helium was used as the carrier gas. On-column injections (2 μl) were performed with the temperature of the injector set at 60°C. The autosampler (7673 A, Hewlett-Packard) was set at 4°C. A temperature gradient was applied of 60–230°C with a heating rate of 20°C min^{-1}. The mass spectrometer was run in the negative ion chemical ionization mode with ammonia as the reagent gas. The ion source temperature was set at 250°C. The ions monitored were m/z 320 and m/z 324-325 for pentafluoropropionyl derivatives of melatonin and internal standard, respectively. These ions were recorded by selective ion monitoring using a computer data system from Hewlett-Packard.

2.4. Standard solutions

Stock solutions of melatonin (and its internal standard when appropriate) were prepared separately at a concentration of 1 mg ml^{-1} in ethanol–water (50:50, v/v) and stored at +4°C for 1 month. Working solutions were prepared for each assay by the appropriate dilution of the stock solutions in water. Standard calibration samples and quality control samples were prepared by spiking either blank human plasma or blank saliva samples with known concentrations of melatonin (from 1 to 100 pg ml^{-1} for GC-MS or from 5 to 200 pg ml^{-1} for LC-RIA) and internal standard (25 pg ml^{-1}).

2.5. Assay validation

Blank plasma and saliva control samples were collected during the daytime, with basal concentrations of melatonin lower than 1 pg ml^{-1}. These biological control media were used without further purification for the preparation of calibration curves.

In the RIA procedures, the specificity of the antibodies was determined by measuring the cross-reactivity of the compounds tested in reference solutions — S 20098, 6-hydroxymelatonin and 3-hydroxylated metabolite of S 20098 (Fig. 1, 3) — and calculating their Abraham indexes.

The LC-RIA method was validated over the concentration range 5–200 pg ml^{-1} with single calibration standards prepared in human plasma at the following concentrations: 5.0, 7.5, 10, 20, 35, 50, 100 and 200 pg ml^{-1}. Calibration curves were constructed by plotting the (B-NSB):(BO-NSB) ratio versus the log of the melatonin concentration, where BO was the control binding determined from a plasma sample in the absence of exogenous melatonin, and NSB was the non-specific binding determined from an antibody-free sample. The standard curve was adjusted with software using the spline function (Packard).

The GC-MS method was validated over the concentration range 1–100 pg ml^{-1}, with single calibration standards prepared in human plasma or saliva at the following concentrations: 1.0, 2.5, 5.0, 10, 25, 50 and 100 pg ml^{-1}. The regression

model was calculated by a weighted least squares regression analysis ($1/y$) based on analyte to internal standard height ratio for different melatonin concentrations. Absolute recovery for extractions from plasma and saliva was estimated by measuring at least three replicates with [^{3}H]*O*-methylmelatonin.

The intra- and inter-assay precision and accuracy were determined for each validation procedure from the analysis of control samples prepared in human plasma or in saliva. For each matrix, three or four different concentrations (three concentrations and the limit of quantitation) were prepared and analysed with a calibration curve during intra-assay analysis (six replicates per concentration) and inter-assay analysis (two replicates per concentration over 5–7 days). Precision was determined as the coefficient of variation (CV%) and accuracy as the percentage relative error from the theoretical concentration (RE%).

For both human plasma and saliva, the stability studies were performed by GC-MS analysis of six replicates of a control sample (20 pg ml^{-1}) stored under different conditions (7 days at 4°C; 7 days at 20°C; 6 months at −20°C) and analysed relative to a calibration curve drown up daily, prepared in the corresponding matrix. The confidence interval limits for the mean concentration obtained for each storage condition were calculated by applying Student's *t*-test (90% level of confidence).

3. Results

3.1. Radioimmunoassays

Two different antisera were tested to determine their specificities towards structural analogues of melatonin (e.g. 6-hydroxymelatonin), S 20098 and one of its main metabolises hydroxylated on the 3 position of the naphthalenic group (Fig. 1, 3).

The cross-reactivities obtained for the different compounds are reported in Table 1. The first antibody, specific for the methoxyindole moiety (Ref. 19540, (1)), gave significant crossreactivities with naphthalenic derivatives; while the second antibody, which is specific for the methoxyindole moiety and the acetamide group, gave a higher specificity for melatonin, with lower cross-reactivities for the naphthalenic compounds. However, the cross-reactivity obtained for S 20098 (6%) limits the use of this antibody for the direct determination of lower levels of melatonin in plasma samples containing S 20098 concentrations up to 200 pg ml^{-1}. An LC-RIA technique was therefore developed.

Table 1
Cross-reactivity of melatonin analogues (percent cross-reaction at ED_{50}) with two commercial antibodies: (1) Ref. 19540 and [^{125}I]2-iodomelatonin as radioligand; (2) Ref. 704-8483 with [^{3}H]*O*-methylmelatonin as radioligand

Compound	Antibody	
	(1)	(2)
Melatonin (Fig. 1, 2)	100	100
6-Hydroxymelatonin	5	0.6
S20098 (Fig. 1, 1)	34	6
3-Hydroxy-S 20098 (Fig. 1, 3)	52	<0.02

3.2. LC-RIA procedure

The chromatographic separation of melatonin derivatives and naphthalenic compounds was optimised on a C8 analytical column with a gradient mobile phase. Melatonin derivatives were co-eluted for between 5 and 8 min and efficiently separated from the naphthalenic derivatives. Using these conditions melatonin analogues were not completely resolved from melatonin. The more specific antibody (HP/S/704-8483) was, therefore, chosen for RIA detection, with [^{3}H]*O*-methylmelatonin as radioligand. In order to increase the sensitivity of the assay, one single analysis of each sample was performed by RIA after fraction collection. An automated solid-phase extraction of the plasma samples was developed on C8 Amprep cartridges for the pretreatment of samples before chromatographic separation. The extraction recovery was determined to be 82%. Under such conditions, the LC-RIA technique has been validated over the range 5–200 pg ml^{-1}, with a

quantitation limit set at 15 pg ml^{-1} per 1 ml of plasma (Table 2). Although accurate results for precision, accuracy and specificity towards S 20098 have been obtained with this procedure, the quantification limit proved to be too high for daytime determination of melatonin. Another alternative was, therefore, studied using GC-MS analysis.

3.3. GC-MS validation

3.3.1. Sample preparation and chromatographic conditions

The derivatization of melatonin with pentafluoropropionic anhydride allows a spirocyclic derivative to be obtained which is specific to melatonin (Fig. 1, 4). This derivative is formed from acylation of melatonin and subsequent thermal rearrangement of the dipentafluoroderivative formed at the beginning of the reaction [21,22]. The derivative gave high sensitivity in the CI mode, with one single ion obtained at $m/z = 320$ corresponding to the loss of two HF residues from the molecular ion. Heptadeuterated melatonin was chosen as internal standard to yield the analogous spirocyclic derivative, with main peaks located at $m/z = 324–325$. A large excess of reagent was used to obtain high derivatization levels for melatonin within 15 min. This led to the formation of corrosive pentafluoropropionic acid as a by-product of the reaction. An automated column-switching system was used [23,24] to prevent the introduction of excess reagents into the capillary columns and into the mass spectrometer. The system consisted of two analytical columns connected by a tee junction switch which could be frozen with liquid carbon dioxide. After chromatographic separation of whole biological extracts on the first column, the tee switch was frozen to trap melatonin and its internal standard before introducing them onto the second analytical column.

An apolar retention gap was connected to the on-column injector to prevent the rapid degradation of the first analytical column. This retention gap was removed after every 100 injections. Solid-phase extraction on C8 cartridges was used as with the LC-RIA procedure for the extraction of human plasma samples. A liquid–liquid extraction procedure was developed with dichloromethane for human saliva, with an ex-

Table 2
Intra- and inter-assay precision and accuracy obtained with LC-RIA and GC-MS for melatonin in human plasma and saliva (LC-RIA inter-assay: $n = 16$ values, GC-MS inter-assay: $n = 10$ values)

	Added conc. (pg ml^{-1})	Intra-assay			Inter-assay		
		Found conc. (mean ± S.D.) ($n = 6$)	CV (%)	RE (%)	Found conc. (mean ± S.D.)	CV (%)	RE (%)
LC-RIA							
Plasma	15	14 ± 2	15.1	−8.9	–	–	–
	30	27 ± 2	8.4	-10.0	33 ± 3	7.4	9.2
	60	65 ± 7	10.2	8.3	64 ± 5	7.7	6.4
	100	115 ± 6	5.2	15.2	103 ± 11	10.7	2.5
GC-MS							
Plasma	1.0	1.1 ± 0.1	10.4	6.0	1.1 ± 0.2	15.1	6.0
	5.0	5.2 ± 0.2	3.8	4.0	5.0 ± 0.8	15.8	0.4
	20	19 ± 1	4.5	−7.5	19 ± 3	14.1	−4.5
	50	49 ± 1	1.0	−1.3	50 ± 7	13.8	0.6
Saliva	1.0	1.1 ± 0.1	8.9	12.0	1.1 ± 0.2	14.5	10.0
	2.5	2.6 ± 0.3	12.2	4.8	2.5 ± 0.2	8.6	-0.3
	20	18 ± 1	3.0	−12.2	20 ± 3	14.8	1.9
	85	74 ± 6	8.0	−12.7	78 ± 8	9.6	8.4

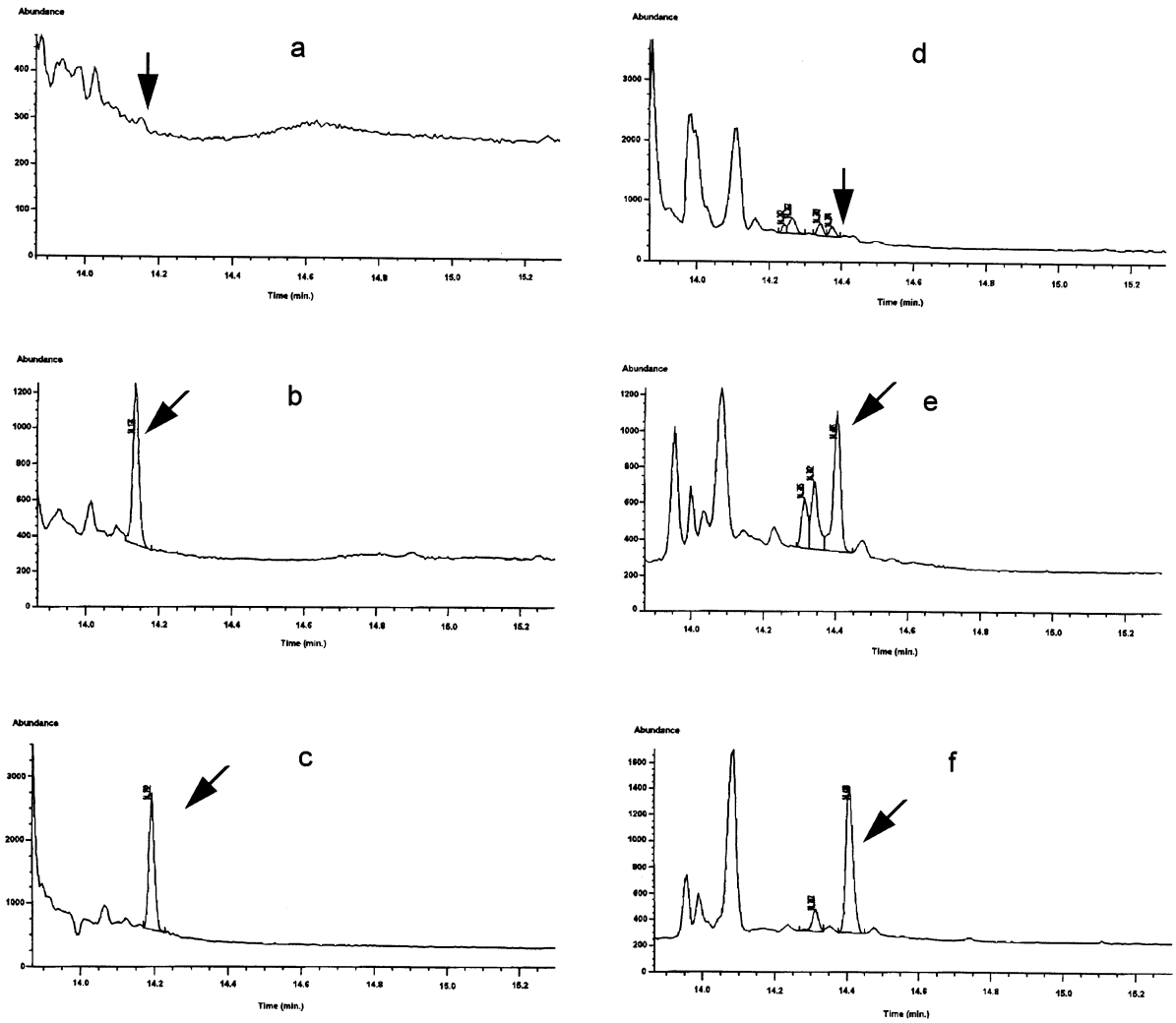

Fig. 2. Typical examples of ion chromatograms at m/z 320 obtained from (a) blank plasma sample, (b) plasma sample spiked with 5 pg ml^{-1} of melatonin, (c) plasma sample from a healthy volunteer treated with 200 mg of S 20098 (21 pg ml^{-1} of melatonin), (d) blank saliva sample, (e) saliva sample spiked with 5 pg ml^{-1} of melatonin, and (f) saliva sample from a healthy volunteer treated with 100 mg of S 20098 (19 pg ml^{-1} of melatonin).

traction recovery of 86%. Each type of extraction proved to be selective for melatonin analysis since no interferences from endogenous compounds or S 20098 and its metabolises were observed in both human plasma and saliva samples (Fig. 2).

3.3.2. Assay validation and stability

The GC-MS procedure was validated over the range 1–100 pg ml^{-1} in both plasma and saliva, with a quantification limit set at 1 pg ml^{-1} in each medium per 1-ml sample.

The linearity was assessed by least squares regression analysis with the following parameters: slope $= 0.039 \pm 0.004$ and coefficient of determination, $r^2 = 0.997 \pm 0.002$ for plasma; slope $= 0.036 \pm 0.005$ and coefficient of determination, $r^2 = 0.999 \pm 0.001$ for saliva ($n = 5$ calibration curves).

The precision and accuracy of the method were determined within 16% at four different levels for intra- and inter-assay analyses (Table 2).

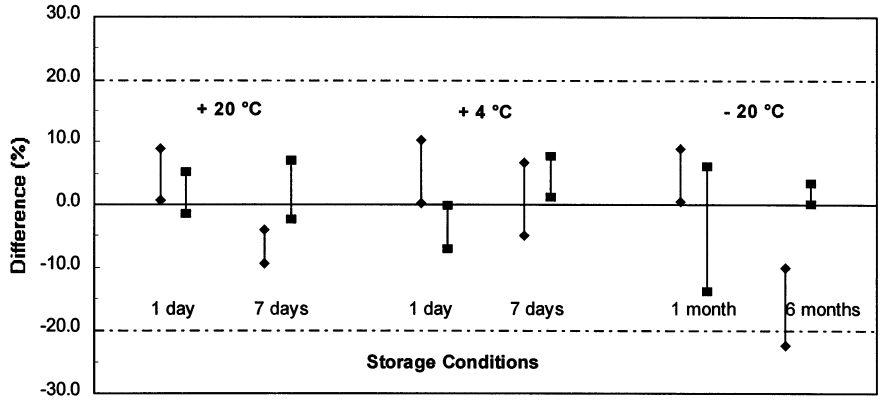

Fig. 3. Confidence interval limits obtained for melatonin stability assay in plasma (◆) and in saliva (■) after storage under different conditions.

Limited data is available for the stability of melatonin in human biological samples [19,33]. The stability of melatonin in plasma and saliva was, therefore, studied following the storage of quality control samples for 7 days at 4 and 20°C, and for 6 months at −20°C. For each storage condition assessed, the 90% confidence limit interval was determined for the difference between the measured concentration and the theoretical concentration (20 pg ml^{-1}) (Fig. 3). The values obtained (all within ±20%) demonstrate the stability of melatonin under the storage conditions outlines both in plasma and saliva.

3.3.3. Assay of clinical samples

The GC-MS procedure was applied to the analysis of melatonin in plasma and saliva samples obtained from clinical studies conducted with S 20098. A profile representative of melatonin plasma concentrations before and after the oral administration of S 20098 (200 mg) to human subjects is shown (Fig. 4). The circadian rhythm of melatonin was quite obvious: endogenous concentrations were lower than 1 pg ml^{-1} around mid-day and a maximum peak concentration was reached between 02:00 and 03:00. Melatonin onset was determined in the evening in saliva before and after the oral administration of S 20098 (100 mg) to healthy subjects (Fig. 4). Similar melatonin concentrations were found in plasma and saliva samples in the placebo or following administration of high doses of S 20098: this result underlines the specificity of the analytical method for the measurement of the hormone after administration of the melatonin agonist S 20098.

4. Discussion and conclusions

The simplicity of immunoassays, together with their sensitivity for the determination of melatonin in biological fluids explains their wide usage and the development of commercially available melatonin kits. However, the main drawback of these assays is the potential lack of specificity towards closely related molecules such as indolic compounds or naphthalenic derivatives including

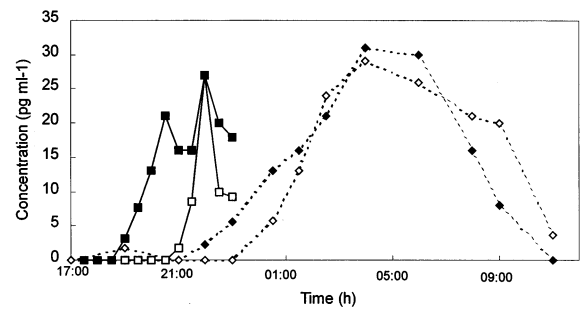

Fig. 4. Melatonin concentration profiles determined in human plasma (--- ◇ --- and --- ◆ ---) and in saliva (--- □ --- and --- ■ ---) with oral administration of S 20098 (◆ and ■) or in placebo subjects (◇ and □).

S 20098. Many attempts to extract melatonin selectively from S 20098 and its metabolites, using either previously described solid-phase extraction procedures [23,24,26] or liquid–liquid extraction methods [25,28,30,31] did not succeed.

An LC-RIA procedure was, therefore, developed to separate melatonin derivatives from naphthalenic compounds. This chromatographic separation, combining RIA detection with an antibody specific to melatonin (Ref. HP/S/704-8483), allowed us to obtain high precision and accuracy for the quantification of melatonin in human plasma, in the range 5–200 pg ml^{-1}. However, the quantification limit was only set at 15 pg ml^{-1}, which limited the analysis of clinical samples containing lower levels of melatonin.

A GC-MS method was subsequently adopted. An automated technique was developed, including automated solid-phase extraction (plasma) or liquid–liquid extraction (saliva) followed by a specific derivatization of the indolic moiety of melatonin and gas chromatographic separation with an automated switching device. This system was preferred over other previously described purification procedures such as washing with alkaline ammonia [28] or injection with a 'falling needle injector' [29] which could not be easily automated.

This technique proved to be highly specific for melatonin, since naphthalenic derivatives such as S 20098 and its metabolites do not react with PFPA. The spirocyclic derivative gave an intense response in mass spectrometry, with the quantification limit for melatonin set at 1 pg ml^{-1} in plasma and saliva, for a linearity range from 1 to 100 pg ml^{-1}.

The method was fully validated in each biological medium, and successfully applied to the specific determination of endogenous melatonin in human plasma and saliva samples collected from clinical studies performed with S 20098.

Immunoassays represent the simplest method for the rapid determination of melatonin in biological samples. However, care should be taken when considering the specificity of this method, especially when samples may contain other melatonin analogues such as the melatonin agonist S 20098.

GC-MS is, therefore, the preferred assay technique for quantification of melatonin in plasma or saliva samples originating from S 20098 clinical studies as it associates both specificity and sensitivity.

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References

[1] G. Bonnefond, L. Martinet, D. Lesieur, G. Adam, B. Guardiola-Lemaître, in: Y. Touitou, J. Arendt, P. Pevet (Eds.), Melatonin and The Pineal Gland — From Basic to Clinical Application, Elsevier, Amsterdam, 1993, pp. 123–126.
[2] T. Azekawa, A. Sano, K. Aoi, H. Sei, Y. Morita, J. Chromatogr. 530 (1990) 47–55.
[3] G. Hernandez, P. Abreu, R. Alonso, C.H. Calzadilla, J. Pineal Res. 8 (1990) 11.
[4] F. Raynaud, P. Pevet, J. Chromatogr. 564 (1991) 103–113.
[5] T. Harumi, H. Akutsu, S. Matsushima, J. Chromatogr. B 675 (1996) 152–156.
[6] M.H. Mills, D.C. Finlay, P.R. Haddad, J. Chromatogr. 564 (1991) 93–102.
[7] J.F. Peniston-Bird, W.L. Di, C.A. Street, A. Kadva, M.A. Stalteri, R.E. Silman, Clin. Chem. 39 (1993) 2242–2247.
[8] M. Hariharan, J.S. Naickar, A.S. Hilibrand, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 1745–1756.
[9] A.A. Vitale, C.C. Ferrari, H. Aldana, J.M. Affanni, J. Chromatogr. B 681 (1996) 381–384.
[10] L.J. Grota, G.M. Brown, Can. J. Biochem. 52 (1974) 196–202.
[11] D.J. Kennaway, R.G. Frith, G. Philippou, C.D. Matthews, R.F. Seamark, Endocrinology 101 (1977) 119–127.
[12] B.J. Lemaître, L. Hartmann, J. Immunol. Methods 32 (1980) 339–347.
[13] J. Arendt, J. Neural. Transm. Suppl. 21 (1986) 11–33.
[14] J. Arendt, L. Paunier, P.C. Sizonenko, J. Clin. Endocrinol. Metab. 40 (1975) 347–350.
[15] S. Fraser, P. Cowen, M. Franklin, C. Franey, J. Arendt, Clin. Chem. 29 (1983) 396–397.
[16] S.M. Yie, E. Johansson, G.M. Brown, Clin. Chem. 39 (1993) 2322–2325.
[17] B. Ferrua, R. Masseyoff, J. Immunoassay 6 (1985) 79–94.
[18] A. Miles, D. Philbrick, S.F. Tidmarsh, D.M. Shaw, Clin. Chem. 31 (8) (1985) 1412–1413.

[19] D.K. Lahiri, D. Davis, M. Adkins, Biochem. Med. Metab. Biol. 49 (1993) 36–50.
[20] S. Chegini, B. Ehrhart-Hofmann, A. Kaider, F. Waldhauser, Clin. Chem. 41 (1995) 381–386.
[21] K.M. Biswas, A.H. Jackson, M. Tehrani, J. Chem. Soc. Chem. Commun. (1982) 765–766.
[22] K.M. Biswas, A.H. Jackson, M.M. Kobaisy, P.V.R. Shannon, J. Chem. Soc. Perkin Trans. 1 (1992) 461–467.
[23] C.R. Lee, H. Esnaud, Biomed. Environ. Mass Spectrom. 15 (1988) 249–252.
[24] C.R. Lee, H. Esnaud, Biomed. Environ. Mass Spectrom. 15 (1988) 677–679.
[25] D.J. Skene, R.M. Leone, I.M. Young, R.E. Silman, Biomed. Mass Spectrosc. 10 (1983) 655–659.
[26] C.W. Tsang, S.F. Pang, C.O. Wong, Adv. Pineal Res. 3 (1989) 181–184.
[27] S. Fraser, P. Cowen, M. Franklin, A.J. Lewy, Clin. Chem. 29 (1983) 1703–1704.
[28] S.P. Markey, A.J. Lewy, Nat. Bur. Stds. Spec. Publ. 519 (1978) 399–404.
[29] J.B. Fourtillan, P. Gobin, B. Faye, J. Girault, J. Biol. Mass Spectrom. 23 (1994) 499–509.
[30] S.A. Best, J.M. Midgley, W. Huang, D.G. Watson, J. Pharm. Biomed. Anal. 11 (1993) 323–333.
[31] O. Beck, P. Pevet, J. Chromatogr. 311 (1984) 1–8.
[32] C.R. Lee, H. Esnaud, Biomed. Environ. Mass Spectrom. 14 (1987) 271–274.
[33] L.J. Wetterberg, J. Neural Transm. Supp. 13 (1978) 289–310.