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Determination of 5-methoxyindoles in pineal gland and plasma samples by high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A liquid chromatographic analysis with electrochemical detection of 5-methoxytryptamine, 5-methoxytryptophol, 5-methoxyindoleacetic acid and melatonin is described. Optimal elution conditions were determined by studying several variables: pH, buffer salt, counter ion and organic modifier. Measurement of 5-methoxyindoles in the pineal gland and plasma of hamsters has been performed after extraction. This method is specific and sensitive, and enables detection of 5-methoxyindoles in a pool of two hamster pineal glands. This is also the first time that these three 5-methoxyindoles have been measured simultaneously in plasma.

INTRODUCTION

Several chromatographic methods have been developed to determine methoxyindoles in physiological samples. One fruitful approach has been to combine reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [1–5]. The use of single cells has enabled hydroxy- and methoxyindoles to be detected in the same sample. However, in the biological tissues studied so far, methoxyindoles are present in much lower amounts than hydroxyindoles. This has caused problems and, in some cases, the authors have been forced to change the sensitivity of their detector in the middle of a run [6]. An additional problem is that, because methoxyindoles are less polar than their corresponding hydroxyindoles, they have long retention times on reversed-phase columns and this diminishes their detection limits.

Dual ED is now extensively used [7]. This method detects compounds on the basis of their oxidation–reduction characteristics. For example, it is known that hydroxyindoles are more readily oxidized than methoxyindoles. Thus, if the first cell is maintained at a lower potential than the second, hydroxyindoles will be totally oxidized at the first detector and the methoxyindoles can be specifically detected in the second cell.

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This procedure of dual detection has recently been used to measure 5-methoxytryptamine (5-MT) in the pineal gland of golden hamsters [8]. However, the method was not optimized and 5-MT eluted very close to the solvent peaks.

The present study assesses the influence of pH, buffer salt, organic modifier and counter ions on the chromatographic parameters of 5-MT, 5-methoxytryptophol (5-ML), 5-methoxyindoleacetic acid (5-MIAA) and melatonin (Mel). An intensity–potential curve of the different 5-methoxyindoles was also determined. The specificity of the technique was evaluated and the recovery of 5-methoxyindoles from cortex and plasma assessed. The limits of detection for standard solutions, brain homogenates and plasma samples were also determined.

EXPERIMENTAL

Chemicals

The 5-methoxyindoles (5-MT, 5-ML, 5-MIAA, Mel) and the other indole and catecholamine derivatives used were obtained from Sigma (St. Louis, MO, U.S.A.), acetonitrile from Baker (Phillipsburg, NJ, U.S.A.), methanol from RPS (Paris, France), disodium hydrogenphosphate, sodium dihydrogenphosphate, sodium acetate and acetic acid from Merck (Darmstadt, Germany) and citric acid, ethylenediaminetetraacetate (EDTA), triethylamine (TEA) and sodium octanesulphonate (OSS) from Fluka (Buchs, Switzerland). Deionized water, filtered through a Milli-Q system (Millipore, Molsheim, France) was used throughout.

Buffers

All buffers were 0.1 *M* and contained 0.01 *M* EDTA. The pH was adjusted with sodium hydroxide, and methanol or acetonitrile were added prior to filtration through Millipore GVWP filters (0.22 μm).

High-performance liquid chromatography

A Waters pump (Model 590) was used at a flow-rate of 1.3 ml/min. The column was a reversed-phase C_{18} XL ODS from Beckman (75 mm × 4.6 mm I.D., 3-μm spherical particles), with a 0.5 cm guard column of the same type. A Rheodyne injection valve (Model 7125) with a 25-μl loop was used for the injections. The detector was a Coulochem 5100 (Environmental Science Assoc., Bedford, MA, U.S.A.). It included a guard cell (Model 5020), which was always maintained at a potential of 1.25 V, and a double detector cell (coulometric–amperometric) (Model 5011). The first cell was maintained at 0.35 V and the second at 0.75 V. Under these conditions the hydroxyindoles were oxidized on the first cell. The integrator was a Waters data module. The calculation method used was the area percent method.

Methods

Influence of pH. The effect of pH was determined in 0.1 *M* phosphate buffer

containing 0.1% (v/v) triethylamine and 14% acetonitrile. The pH was modifed between 3.0 and 5.7.

Influence of the salt. Different 0.1 *M* salts were tested. They all contained 0.1% (v/v) triethylamine and 14% acetonitrile. Citrate (pH 3.25), acetate, phosphate, phosphate–citrate buffers, all at pH 4.6, were used.

Influence of the nature and proportion of the organic modifier. This was tested in 0.1 *M* phosphate buffer containing 10–20% methanol or acetonitrile.

Influence of the presence of counter ions. The effect of counter ions was determined in 0.1 *M* phosphate buffer. The influence of alkaline [0.1% (v/v) triethylamine] or acidic (0.2 m*M* sodium octane sulphonate) counter ion, alone or in combination, on the retention of the different methoxyindoles was determined.

Intensity potential. The detector gain was set at 1 × 50. The potential was increased in 0.05-V steps from 0.40 to 0.85 V. A mixture containing 2.5 ng of all four methoxyindoles (5-MT, 5-ML, 5-MIAA, Mel) was injected at each potential.

Specificity of the technique. In order to evaluate potential interferences, 3-methoxytyramine, serotonin, N-acetylserotonin, 5-hydroxytryptophol, 5-hydroxyindoleacetic acid, 5-methoxytryptophan, 6-hydroxymelatonin, 5-methoxytryptoline and tryptoline were injected with a mixture of the 5-methoxyindoles.

Biological samples

Brain homogenates. Cortexes from golden hamsters, known not to contain any 5-methoxyindoles, were homogenized in 0.1 *M* perchloric acid. External standards were added to the medium prior to sonication. The influence of the antioxidants EDTA (0.01 *M*), ascorbic acid (0.1%), sodium metabisulphate (0.01 *M*) and cysteine (0.01 *M*) on the recoveries of the methoxyindoles was tested. The samples were filtered through HV filters (Millipore) prior to HPLC injection. Detection limits were determined by adding external standards to the samples, and the inter- and intra-assay coefficients of variation (C.V.) at concentrations of 20, 50, 100 and 500 pg were assessed. For each concentration of standard, five samples were measured.

Male golden hamsters kept under long photoperiod (14 h light, 10 h dark, with lights off at 18:00) at 20°C were killed at 16:00 and their pineal glands removed. The animals were treated with monoamine oxidase inhibitors as this was known to increase the amount of 5-MT [8]. Two animals were treated with 2.5 mg/kg deprenyl intraperitoneally, an inhibitor of monoamine oxidase B, 1.5 h before being sacrified, three animals were treated with 100 mg/kg pargyline intraperitoneally, a non-specific monoamine oxidase inhibitor, 24 h before being killed, and three animals were untreated.

Plasma. Different concentrations of external standards (10, 50, 100, 200, 500 and 1000 pg) were added to plasma from golden hamsters and extracted by different procedures. For each concentration of standard, four samples were extracted.

For extraction under neutral conditions, five volumes of dichloromethane were added to the samples and the tubes were vortex-mixed for 25 s.

For extraction under alkaline conditions, the same procedure as for neutral extraction was used with the addition of 5% (v/v) NaOH (1 *M*) to the samples.

For alkaline extraction with addition of antioxidants, the neutral extraction procedure was used with the addition of 0.5 m*M* $Na_2S_2O_5$, 0.5 m*M* EDTA, 0.1% ascorbate oxidase and 5% (v/v) NaOH (1 *M*).

For neutral extraction followed by alkaline extraction, five volumes of dichloromethane were added to the samples containing 0.5 m*M* $Na_2S_2O_5$, 0.5 m*M* EDTA and 0.1% ascorbate oxidase. The tubes were vortex-mixed for 25 s, then 5% (v/v) NaOH (1 *M*) was added and the tubes were again vortex-mixed for 25 s.

In all cases, the samples were centrifuged for 10 min at 1200 *g*, the aqueous phase was aspirated, and four volumes of dichloromethane were evaporated under vacuum. The residues were dissolved in 25 μl of water, and 20 μl were injected into the HPLC loop.

The detection limit was determined using hamster plasma that had been charcoal-stripped to remove the endogenous 5-methoxyindoles. Standard solutions of 5-methoxyindoles were added to the blank plasma and extracted. Inter- and intra-assay C.V. were also determined by the addition of external standards to a pool of plasma (10, 50, 100 and 500 pg).

RESULTS AND DISCUSSION

Optimization of the HPLC method

Among the 5-methoxyindoles, the capacity factor of only 5-MIAA was affected by the pH, presumably as a result of an acid–base equilibrium. A clear separation of the different compounds was achieved whenever the pH was higher than 4.3.

The different buffer salts that were tested did not affect the chromatographic parameters of the 5-methoxyindoles (data not shown).

Altering the percentage of the organic modifier did not change the order of elution of the 5-methoxyindoles studied. However, since methanol clearly increased the pressure in the system, acetonitrile was used. Good separation was achieved using acetonitrile between 10 and 16%.

The effect of different counter ions on the capacity factors is shown in Table I. Triethylamine (TEA), an alkaline counter ion, increased the retention time of the acid (5-MIAA) but slightly decreased the retention time of the amine (5-MT). In contrast, the acidic counter ion sodium octanesulphonate (OSS) increased the retention time of the amine (5-MT), but slightly decreased the retention time of the acid (5-MIAA). TEA was used in order to avoid any interaction of the amino groups of 5-MT with the column, which would result in tailing peaks. OSS was used to increase the retention time of the amine in order to separate it from the solvent peaks. Thus, for optimal elution of 5-MT, both counter ions (TEA and

TABLE I

EFFECTS OF DIFFERENT COUNTER IONS ON THE CAPACITY FACTORS OF METHOXYINDOLES

Conditions: phosphate–citrate (pH 4.6), 0.1 m*M* EDTA, 0.1% TEA, 0.2 m*M* OSS.

Compound	Capacity factor			
	Without counter ion	With TEA	With OSS	With TEA + OSS
5-MT	6.8	5.6	8.8	7.7
5-MIAA	12.8	13.9	12	13
5-ML	20.1	20.1	20.1	20.2
Mel	23.7	23.7	23.7	23.7

OSS) were used. The simultaneous use of acidic and alkaline counter ions has previously been described for the determination of catecholamines and their metabolites [9].

Based on the above studies, 0.1 *M* buffer (pH between 4.3 and 5.0) containing 10–16% acetonitrile was the optimum for the determination of 5-ML, 5-MIAA and Mel. However, when 5-MT was also measured, counter ions (TEA and OSS) were added to the buffer.

Our results show that the acid–base characteristics of the molecules are affected by the pH and the presence of counter ion. Other studies on the chromatography of hydroxyindoles and monoamines have shown similar results [10,11].

From the intensity–potential curve of the different 5-methoxyindoles a potential of 0.75 V was chosen (Fig. 1).

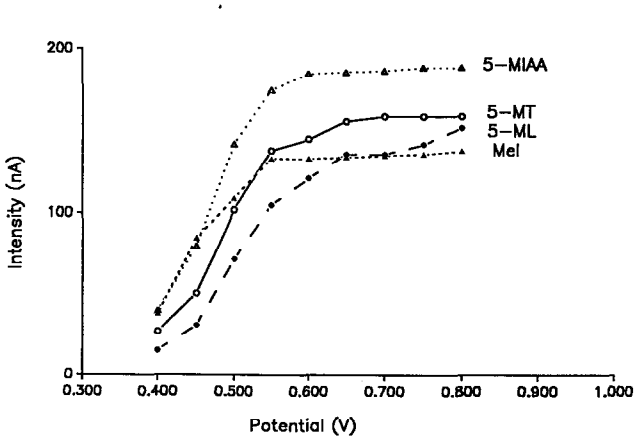

Fig. 1. Intensity–potential curve for the 5-methoxyindoles. Buffer, 0.1 *M* phosphate–citrate (pH 4.6) containing 0.1 m*M* EDTA, 0.1% (v/v) triethylamine and 14% acetonitrile.

Under our chromatographic conditions, the hydroxyindoles were always oxidized at the first cell. The methoxylated derivatives of catecholamines and carbolines tested did not interfere with the methoxyindoles (data not shown).

Fig. 2 shows a chromatogram of the four methoxyindoles. The detection limits for the standard solutions were 1.5 pg for 5-MT, 3 pg for 5-ML and 5-MIAA and 4 pg for Mel.

Sample preparation

The effects of light and antioxidants on the recoveries of the 5-methoxyindoles are presented in Table II. Only 5-MT was light-sensitive, and it was also subject

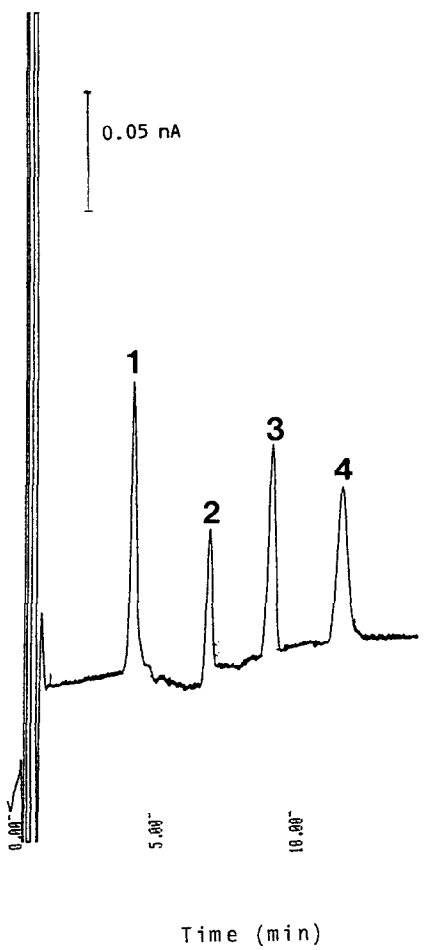

Fig. 2. Chromatogram of a standard solution containing 100 pg of 5-methoxytryptamine (1), 60 pg of 5-methoxyindoleacetic acid (2), 100 pg of 5-methoxytryptophol (3) and 140 pg of melatonin (4). Buffer, 0.1 *M* phosphate–citrate buffer containing 0.1 m*M* EDTA, 0.1% (v/v) triethylamine, 0.2 m*M* sodium octanesulphonate and 14% acetonitrile.

TABLE II

EFFECT OF DIFFERENT ANTIOXIDANTS ON THE RECOVERIES OF 5-METHOXYINDOLES IN GOLDEN HAMSTER CORTEX

Conditions: phosphate–citrate (pH 4.3) containing 13% acetonitrile, 0.1 m*M* EDTA, 0.1% TEA and 0.2 m*M* OSS. Values are percentages of the original concentrations (n = 20).

Compound	Recovery (%)				
	$HClO_4$,	$HClO_4$, dark	$HClO_4$, dark, ascorbic acid, EDTA	$HClO_4$, dark, cysteine, EDTA	$HClO_4$ dark, $Na_2S_2O_5$, EDTA
5-MT	30–45	45–56	76–102	92–101	94–100
5-ML	60–80	40–82	40–68	51–62	56–77
5-MIAA	103–107	102–104	100–101	96–101	96–101
Mel	96–101	97–100	96–102	92–105	96–103

to oxidative degradation: the recoveries of 5-MT increased by 100% on addition of antioxidants. At the potential used for our determination, ascorbic acid and cysteine were oxidized and generated large peaks in the chromatogram. Sodium metabisulphite was thus the antioxidant of choice. The limits of sensitivity of the 5-methoxyindoles in cortex homogenates and the C.V. are presented in Table III.

TABLE III

DETECTION LIMITS AND INTER- AND INTRA-ASSAY C.V. DETERMINED IN MALE GOLDEN HAMSTER CORTEX HOMOGENATES AND EXTRACTED PLASMA

Compound	Detection limit (n = 5)	C.V. (n = 15) (%)	
		Inter-assay	Intra-assay
Standards			
5-MT	1.5		
5-ML	3		
5-MIAA	3		
Mel	4		
Cortex (pg per sample)			
5-MT	3	5–8	3–5
5-ML	4	5–9	4–6
5-MIAA	4	5–9	5–7
Mel	8	5–10	4–6
Plasma (pg/ml)			
5-MT	5	8–12	3–6
5-ML	8	9–12	4–8
5-MIAA	–	–	–
Mel	10	12–13	7–12

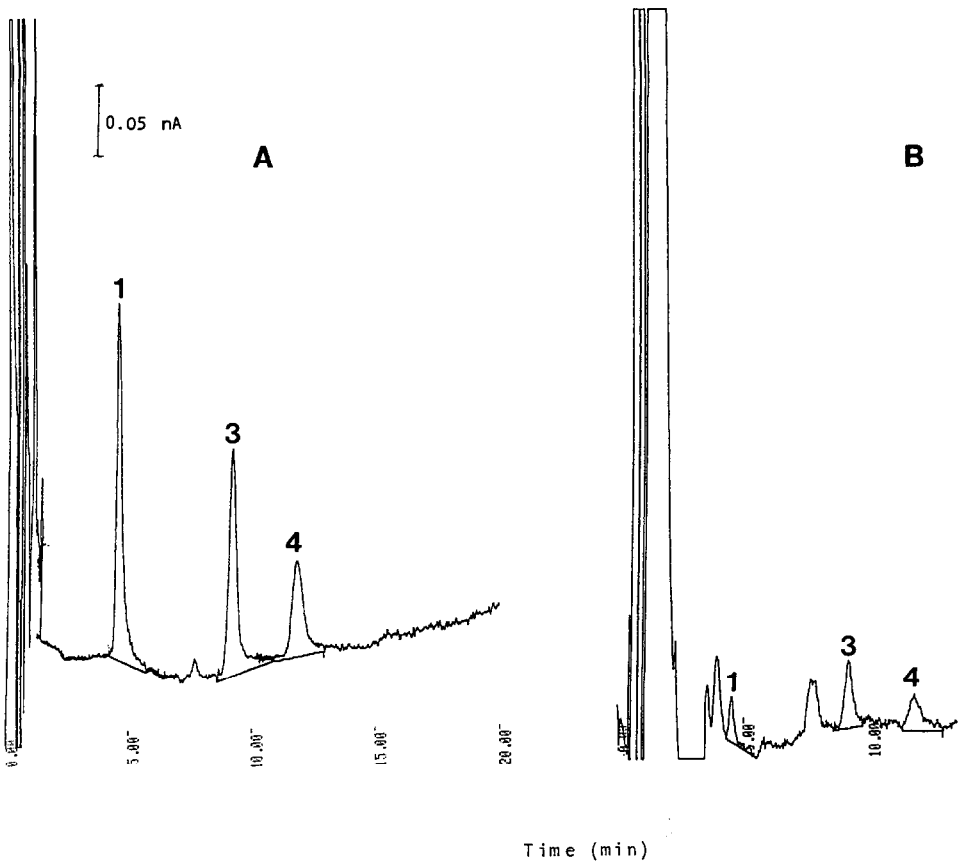

Fig. 3. Chromatograms of (A) a standard solution containing 200 pg of 5-methoxytryptamine (1), 5-methoxytryptophol (3) and 150 pg of melatonin (4) and (B) a pool of two pineal glands of golden hamsters after administration of deprenyl 1.5 h before sacrifice. Buffer, 0.1 *M* phosphate–citrate (pH 4.3) containing 0.1% (v/v) triethylamine, 0.15 m*M* sodium octanesulphonate and 14% acetonitrile.

Fig. 3 shows a chromatogram of a standard solution of methoxyindoles and a chromatogram of a pool of two pineal glands of golden hamsters pretreated with the monoamine oxidase B inhibitor, deprenyl. The concentrations of the different methoxyindoles that were measured in male golden hamsters untreated or pretreated with pargyline or deprenyl are presented in Table IV.

The concentrations of 5-methoxyindoles were also measured in plasma samples. As 5-MT is light-sensitive, solvent extraction was chosen in preference to using extraction columns because it is easier to perform in the dark. In addition, following column extraction, the extract would have to be concentrated in order to detect the low concentrations of 5-methoxyindoles. Therefore, solvent extraction presently seems to be the best procedure to purify and concentrate the sample prior to injection. As shown in Table V, it was possible to extract 5-MT, 5-ML

TABLE IV

PINEAL 5-METHOXYINDOLE CONCENTRATIONS IN MALE GOLDEN HAMSTERS UNTREATED OR TREATED WITH MONOAMINE OXIDASE INHIBITOR

Sample	Concentration (pg per pineal)			
	5-MT	5-MIAA	5-ML	Mel
Untreated (n = 3)	3	120	12	23
Treated with deprenyl (n = 2)	13	–[a]	18	31
Treated with pargyline (n = 3)	96	–[a]	N.D.[b]	87

[a] Not measured.
[b] Not detectable.

and Mel from a sample by neutral and then alkaline extraction. The detection limits of the method in charcoal-stripped plasma, as wel as the C.V., are shown in Table III. With this technique, 5 pg of 5-MT, 8 pg of 5-ML and 10 pg of Mel could be detected in hamster plasma. A chromatogram of a plasma extract 1 h after injection of 25 μg of 5-MT to male golden hamsters in shown in Fig. 4.

Our HPLC technique is thus sensitive enough to detect picogram levels of 5-methoxyindoles in hamster pineal gland and plasma. 5-MT [8], 5-MIAA [5,6], 5-ML [5,6] and Mel [5,6,8] have previously been measured by HPLC in hamster [8] and rat [5,6] pineal glad homogenates, with a sensitivity comparable with what we have achieved. However, using the presently described technique, all four 5-methoxyindoles can be measured simultaneously in pools of two golden hamster pineal glands or in a single rat pineal gland. Mefford and co-workers [5,6]

TABLE V

EFFECTS OF DIFFERENT EXTRACTION PROCEDURES ON THE RECOVERIES OF 5-METHOXYINDOLES EXTRACTED FROM HAMSTER PLASMA

Alkaline procedure, 100 μl sodium hydroxide (1 M) per ml of plasma. Antioxidant, $Na_2S_2O_5$ (0.1 mM). Values are percentages of the original amount (n = 24).

Compound	Recovery (%)			
	Neutral	Alkaline	Alkaline antioxidant	Neutral alkaline antioxidant
5-MT	0	27–31	58–70	57–71
5-ML	88–98	12–14	12–14	88–98
Mel	92–102	88–97	88–99	96–102

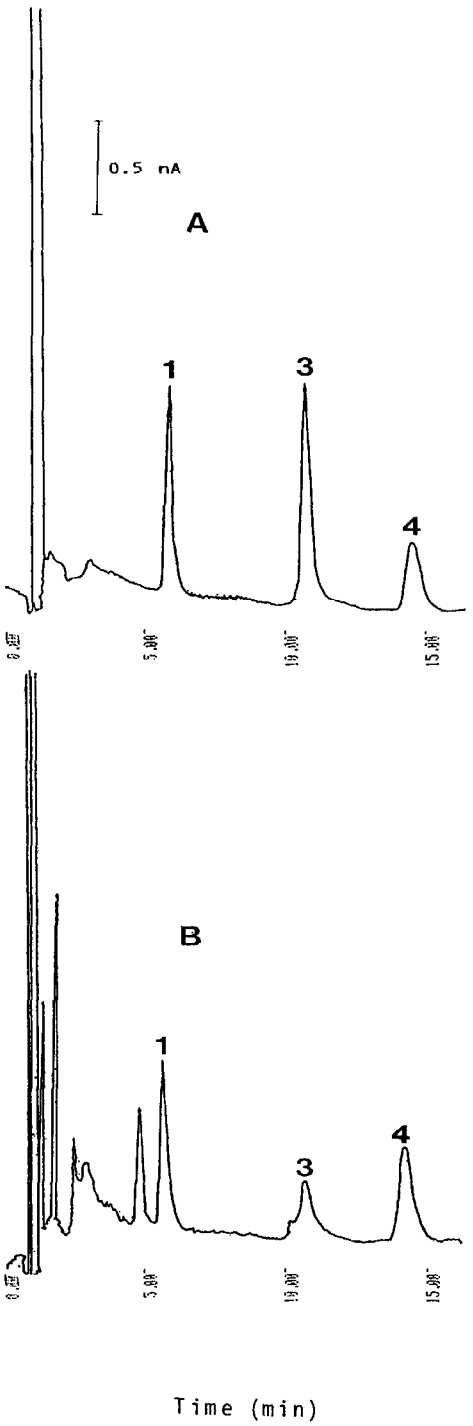

Fig. 4. Chromatograms of (A) a standard solution containing 500 pg of 5-methoxytryptamine (1), 5-methoxytryptophol (3) and 300 pg of melatonin (4) and (B) extracted plasma of golden hamster, 1 h after receiving 25 μg of 5-MT subcutaneously. Buffer, 0.1 *M* phosphate–citrate (pH 4.3) containing 0.1% (v/v) triethylamine, 0.15 m*M* sodium octanesulphonate and 12% acetonitrile.

reported an HPLC technique by which they measured 5-ML, 5-MIAA and Mel in a single chromatographic run. However, in their study, 5-ML and Mel eluted very close together and precision data were not given. Under our conditions, 5-MT, 5-ML, 5-MIAA and Mel are clearly separated with good reproducibility.

5-MT has only been measured by HPLC by Galzin *et al.* [8], 5-MT eluting very close to the solvent peaks. Our use of an acidic counter ion in combination with an alkaline counter ion has eliminated this problem which, in turn, has improved the reproducibility.

The concentrations of 5-MT measured in hamster pineals following pargyline administration are in agreement with that reported by Galzin *et al.* [8], a result that has been validated by gas chromatography–mass spectrometry. The levels of 5-ML measured in the hamsters pineals with our HPLC method are also in accordance with the amounts measured by radioimmunoassay [12].

To our knowledge this is the first report describing the simultaneous determination of several 5-methoxyindoles in plasma. The plasma extraction procedure has been optimized and the reproducibility checked. Moreover, plasma Mel and 5-ML levels measured using this HPLC technique correlate well with the concentrations that have been measured in our laboratory using RIA [13,14].

In conclusion, we present here a sensitive, specific and reproducible HPLC method for the simultaneous determination of different 5-methoxyindoles, including 5-MT, in biological tissues and fluids.

REFERENCES

1 M. A. A. Namboodiri, D. Sugden, I. N. Mefford and D. C. Klein, in S. Parvez (Editor), *Methods in Biogenic Amine Research*, Elsevier, New York, 1983, p. 549.
2 P. B. Foley, K. Cairncross and A. Foldes, *Neurosci. Biobehav. Rev.*, 10 (1986) 273.
3 J. Lee Chin, *J. Chromatogr.*, 428 (1988) 206.
4 G. Hernandez, P. Abreu, R. Alonso and C. H. Calzadilla, *J. Pineal Res.*, 8 (1990) 11.
5 I. N. Mefford, P. Chang, D. C. Klein, M. A. A. Namboodiri, D. Sugden and J. Barchas, *Endocrinology*, 113 (1983) 1583.
6 I. N. Mefford and J. Barchas, *J. Chromatogr.*, 181 (1980) 187.
7 S. A. Mc Clintock and W. C. Purdy, in S. Parvez (Editor), *Progress in HPLC, Electrochemical Detection in Medicine and Chemistry*, UNU, Utrecht, 1987, p. 37.
8 A. M. Galzin, M. T. Eon, H. Esnaud, C. R. Lee, P. Pévet and S. Z. Langer, *J. Endocrinol.*, 118 (1988) 389.
9 P. Wester, J. Gottfries, K. Johansson and F. Klintebach, *J. Chromatogr.*, 415 (1987) 261.
10 P. Heregodt, Y. Michotte and E. Ebinger, *J. Chromatogr.*, 345 (1985) 33.
11 J. H. Trouvin, A. Gardier, G. El Gemayel and C. Jacquot, *J. Liq. Chromatogr.*, 10 (1987) 261.
12 D. J. Skene, P. Pévet, B. Vivien-Roels, M. Masson-Pévet and J. Arendt, *J. Endocrinol.*, 114 (1987) 301.
13 F. Raynaud, J. L. Miguel, B. Vivien-Roels, M. Masson-Pévet and P. Pévet, *J. Endocrinol.*, 121 (1989) 507.
14 D. J. Skene, B. Vivien-Roels and P. Pévet, *Neurosci. Lett.*, 108 (1990) 138.