Journal of Chromatography, 427 (1988) 326–330
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4146

Note

Automatic determination of serotonin in biological fluids by liquid chromatography

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(First received July 23rd, 1987; revised manuscript received February 2nd, 1988)

Serotonin is located primarily in the enterochromaffin cells of the intestine, the seronergic neurons of the brain and the platelets of blood. For clinical purposes, measurement of serotonin is important for the diagnosis of pathological phenomena such as depression [1], carcinoid syndrome [2] and essential hypertension [3]. Several methods have been described for the determination of serotonin in biological fluids including fluorimetric techniques [4–6], gas chromatography–mass spectrometry [7], radioimmunology [11] and high-performance liquid chromatography (HPLC) [8–10]. However, they lack sensitivity and specificity, or require several extraction steps for concentration and purification of the samples. For routine clinical analysis these methods are laborious and time-consuming. Therefore we have developed a new method based on HPLC and fluorimetric detection without sample extraction for an automatic clinical analysis of serotonin in biological fluids.

EXPERIMENTAL

Apparatus and chromatographic conditions

The chromatographic system consisted of two 655A-12 liquid chromatograph pumps, an L5000 LC controller, a 655A-40 autosampler, a D2000 integrator (Merck, Darmstadt, F.R.G.) and an ELV 7000 electromagnatic valve (Krannich, Göttingen, F.R.G.). A 25 mm × 4 mm I.D. pre-column and a 125 mm × 4 mm I.D. analytical column packed with LiChrospher RP 18e (end-capped, 5 μm) was employed (Merck). The switching diagram is given in Fig. 1. The HPLC mobile phase consisted of 5 m*M* octanesulphonic acid sodium salt (Fluka, Buchs, Swit-

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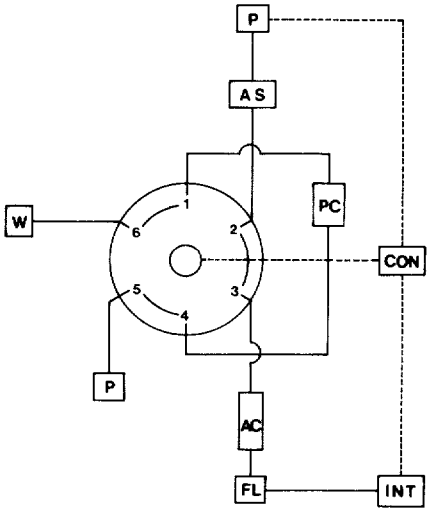

Fig. 1. Diagram of the HPLC column-switching system in the "stripping mode" for serotonin determination. AC = analytical column; AS = autosampler; CON = controller; FL = fluorescence detector; INT = integrator; P = pump; PC = pre-column; W = waste.

zerland) in 0.1 *M* sodium dihydrogenphosphate–phosphoric acid buffer (pH 3.0) containing 25% methanol. The flow-rate was 1.0 ml/min, and the column effluent was monitored by an F1000 fluorescence spectrometer (Merck) with excitation at 285 nm and emission at 345 nm.

Sample preparation

Blood and urine samples were centrifuged at 4000 *g* before analysis except EDTA blood samples, which were centrifuged at 500 *g*. To each of the micro-sampling tubes, containing 250 μl of serum, plasma or urine, were added 50 μl of ice-cold 3.4 *M* perchloric acid. The mixture was vortexed, cooled for 5 min at $-20\,^{\circ}$C and centrifuged for 3 min at 10 000 *g*. The supernatant (100 μl) was diluted with 900 μl of mobile phase, and 40 μl of this mixture were injected by an autosampler for analysis. Standard curves were obtained for serotonin by external calibration and performed as described above.

RESULTS AND DISCUSSION

For the quantification of serotonin in biological fluids an automatic HPLC system was used (Fig. 1). The system is controlled by an L5000 LC controller, which activates the valve and integrator. The pretreated and diluted sample is injected by an autosampler, which activates the controller. Within a 12-min cycle, the autosampler injects a 40-μl aliquot of the samples on a pre-column, and unretained substances pass towards the analytical column. The electromagnetic valve switches over after 1.5 min and the strongly retained body fluid components are back-flushed from the pre-column for 8.5 min by a second HPLC pump with the same mobile phase. This "stripping mode" enables the elution of strongly re-

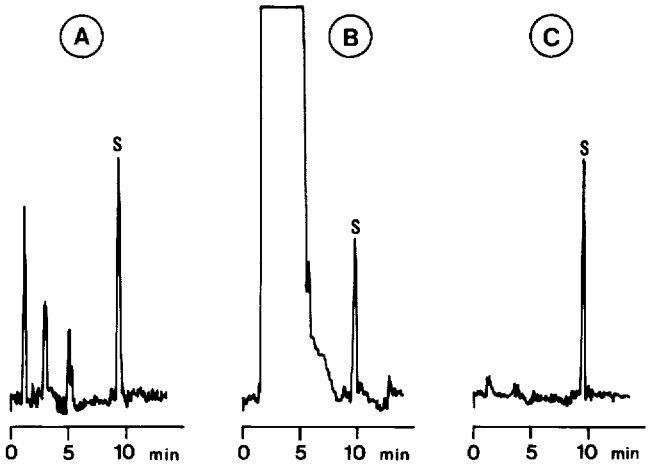

Fig. 2. Chromatograms of diluted and pretreated serotonin (S) samples on an RP 18e column. (A) Serum sample (213 ng/ml); (B) urine sample (133 ng/ml); (C) calibration sample (200 ng/ml).

tained body fluid substances from the pre-column, which otherwise would interfere with the next serotonin analysis. A great advantage of this automatic HPLC system is that time-consuming gradient elution profiles can be avoided. Serotonin and other unidentified components, which are not retained by the pre-column, are separated on an analytical column and monitored fluorimetrically.

Several experiments showed that a mobile phase containing an ion-pair reagent is advantageous for the separation of serotonin from other physiological contaminants. Mobile phases of various pH and without ion-pair reagents were tested, but gave unsatisfactory results. Other body fluid components interfered or overlapped with the serotonin peak. In other experiments, several ion-pair reagents with different aliphatic chain lengths were tested, to find the optimal elution and separation conditions. Ion-pair reagents with increasing chain lengths, such as butane-, pentane-, hexane-, heptane- or octanesulphonic acid increased the retention of serotonin on a reversed-phase column. The highest efficiency and reproducibility and the shortest analysis times were obtained when octanesulphonic acid was used.

Direct injection of urine, serum or plasma samples showed unsatisfactory separation of serotonin, and impurity problems resulted. Therefore a simple and efficient pretreatment was chosen. Instead of the time-consuming extraction steps, a protein precipitation step was performed. Perchloric acid precipitation was sufficient to eliminate interfering body fluid components. Additionally, fluorimetric detection allowed a selective measurement of serotonin.

For serotonin quantification in serum and plasma, only a few early eluting impurities were observed (Fig. 2). Even with urine, which is the most difficult and complex matrix, reliable serotonin determinations were possible with this system (Fig. 2). Serotonin concentrations even below 10 ng/ml are easily detectable with this method. Tables I and II demonstrate recovery, precision and stability studies on serotonin concentrations in urine, plasma and serum. This newly developed method shows, for matrix-dependent and matrix-independent sam-

TABLE I

MATRIX-DEPENDENT AND MATRIX-INDEPENDENT SEROTONIN RECOVERY DATA OF DIFFERENT BODY FLUIDS

C.V. = coefficient of variation.

Sample	Concentration (ng/ml)	n	Recovery (mean ± C.V.) (%)
Standard	100	40	99.29 ± 1.15
	200	40	98.95 ± 1.20
Serum	200	20	97.98 ± 2.16
Plasma	200	20	94.96 ± 4.25
Urine	195	15	96.53 ± 3.57

ples, reproducible data in series as well as for between-day precision (Table II). Identical results were obtained by standard addition and external standard calibration. The calibration plots were linear in the range 10–800 ng/ml serotonin. The method is suitable for the determination of serotonin in biological fluids, because normal levels (serotonin in serum < 200 ng/ml, in EDTA plasma 100–300 ng/ml, in urine < 200 ng/day) as well as pathological serotonin levels can be measured. No interferences from drugs have been observed for serotonin determinations in all tested biological fluids by taking advantage of ion-pair chromatography and selective fluorimetric detection.

The sensitivity of this method can be increased by injecting larger sample volumes and by a lower dilution of the sample. Furthermore, the assay time for serotonin determinations in serum and plasma can be shortened by increasing the concentration of methanol in the mobile phase, because in these body fluids, interfering substances were not observed (Fig. 2). Conversely, this elution profile was needed for urine analysis because there are too many interfering substances present.

In summary, this new automated method opens up the possibility of analysing serotonin in urine, serum, and plasma within 12 min without sample extraction

TABLE II

PRECISION AND STABILITY DATA OF SEROTONIN DETERMINATIONS IN DIFFERENT BODY FLUIDS

Series, $n = 20$; day-to-day, $n = 31$; C.V. = coefficient of variation.

Sample	Concentration		Retention time	
	C.V. series (%)	C.V. day-to-day (%)	C.V. series (%)	C.V. day-to-day (%)
Standard	1.86	2.96	0.61	1.95
Serum	2.95	–	0.69	–
Plasma	3.05	–	0.70	–
Urine	3.89	–	0.74	–

procedures. A perchloric acid precipitation step is sufficient to eliminate interfering body fluid components. Furthermore, gradient elution can be avoided by a column-switching technique in the stripping mode. Fluorimetric detection allows specific and sensitive determination of serotonin.

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