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Note

Determination of plasma serotonin by high-performance liquid chromatography with pre-column sample enrichment and fluorimetric detection

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In addition to the role played by serotonin (5-HT) in physiological functions such as sleep regulation [1] and behaviour [2], or in pathological conditions such as depression [3] and carcinoid syndrome [4], this biogenic amine is also implicated in haemostasis, thrombosis and cardiovascular diseases [5]. Specific clinical–biochemical or metabolic studies require the accurate measurement of the intraplatelet and/or extraplatelet pool of 5-HT, so a simple, sensitive and rapid methodology for the determination of 5-HT is of great interest.

Several methods have been reported for the determination of plasma 5-HT, including fluorimetric assays [6–9] and radioimmunological [10,11] or radioenzymic [12] techniques. However, conventional fluorimetric methods lack specificity and sensitivity, and radioisotopic assays are expensive and too time-consuming for routine analysis. More recently, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been applied to the analysis of 5-HT in plasma [13–18]. HPLC–ED has proved to be a sensitive, rapid and precise technique for the determination of indoleamines; however, analysis of samples with a complex matrix or of low amine content requires time-consuming clean-up and pre-concentration steps before analytical chromato-

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graphy; microcolumn ion exchange [13,18] or solvent extraction [15] of the indoleamine have been widely used.

This paper describes a suitable method for the assay of 5-HT in platelet-poor plasma (PPP) and in platelet-rich plasma (PRP); it involves ion-exchange HPLC with on-line sample enrichment and fluorimetric detection. The methodology includes acid extraction of 5-HT from PPP and PRP by trichloroacetic acid, direct injection of large volumes (0.5–2 ml) of the extract into a Guard-Pak C_{18} preconcentration cartridge connected on-line to the analytical column and 5-HT separation by ion-exchange HPLC.

EXPERIMENTAL

Chemicals

Serotonin creatinine sulphate and N-methyl-5-hydroxytryptamine oxalate (internal standard, I.S.) were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were analytical- or HPLC-grade products from Inalco (Milan, Italy). HPLC-grade water was obtained by feeding distilled water to a Norganic water purification system obtained from Millipore/Waters (Milan, Italy).

The stock solution of standard 5-HT was prepared by dissolving 22.8 mg of serotonin creatinine sulphate in 100 ml of water to give 100 μg free base (0.57 mM). The stock solution of I.S. was prepared at 100 μg/ml (0.53 mM) as free N-methylserotonin in water. These solutions were stored at 4°C in the dark and were freshly prepared every two weeks. Working standard solutions of 5-HT and I.S. were obtained at 2 ng/ml by diluting the stock solutions with 2% trichloroacetic acid solution containing 0.1% (w/v) sodium disulphite and 0.1% (w/v) disodium ethylenediaminetetraacetate.

Apparatus

The chromatograph consisted of a Model Clar 002 pump (Violet, Rome, Italy); a Model FC 530 spectrofluorimeter (Shimadzu, Kyoto, Japan), equipped with a 12-μl flow-cell and set at an excitation wavelength of 285 nm and at an emission wavelength of 344 nm; a Model 191 injection valve (Negretti & Zambra, Southampton, U.K.). The loop of the sample injector was replaced with a Guard-Pak Module (Millipore/Waters) fitted with a C_{18} cartridge. The output of the detector was connected to a Model C-R1B electronic integrator (Shimadzu). Chromatographic separations were performed using a Partisil SCX column (250×4.6 mm I.D., 10 μm particle size).

Sample preparation

5-HT levels in PRP and PPP were determined in fifteen apparently healthy male adults (27.07 ± 4.6 years old, mean ± S.D.). Venous blood samples (10 ml) were drawn from the subjects in the fasting state at 09:00 a.m. in plastic tubes containing 0.1 ml of 15% (w/v) disodium ethylenediaminetetraacetate solution. After mixing, blood specimens were kept in ice, and PRP and PPP were prepared, essentially according to the method of Picard et al. [17]. PRP was obtained by

centrifugation of the blood at 160 *g* for 10 min at 4°C. A platelet count on PRP was obtained using a Model FN Coulter Counter. A 1-ml aliquot of PRP was transferred into a plasic tube containing 1 mg of sodium disulphite and taken for assay, or stored at −35°C until used for assay. A 2-ml aliquot of PRP was centrifuged at 6000 *g* for 10 min at 4°C, and the supernatant PPP was carefully decanted and used for analysis or stored at −35°C in a plastic tube containing 2 mg of sodium disulphite.

To a 1-ml aliquot of PPP sample in a 5-ml polypropylene centrifuge tube were added 40 μl of a 100 ng/ml I.S. solution, 0.46 ml of a 0.9% (w/v) sodium chloride solution and 0.5 ml of a 12% (w/v) trichloroacetic acid solution, containing 0.1% (w/v) sodium disulphite. The sample was vortexed for 30 s and centrifuged at 15 000 *g* for 5 min at 4°C. The clear supernatant was kept in ice, and a 0.5-ml aliquot was used for direct injection into the chromatograph, according to the procedure described below.

To 0.1 ml of PRP sample in a 5-ml polypropylene centrifuge tube were added 0.55 ml of a 0.9% (w/v) sodium chloride solution, 0.1 ml of a 100 ng/ml I.S. solution and 0.25 ml of a 12% (w/v) trichloroacetic acid solution, containing 0.1% (w/v) disodium ethylenediaminetetraacetate and 0.1% (w/v) sodium disulphite. The sample was vortexed for 30 s and centrifuged at 15 000 *g* for 5 min at 4°C. The supernatant was placed in ice, and 0.1 ml was used for the HPLC analysis.

Chromatographic conditions

The mobile phase used for the isocratic elution was 0.05 *M* citric acid–acetonitrile (80:20, v/v). This mixture was adjusted to pH 3.20 ± 0.02 at 25°C with 4 *M* sodium hydroxide and degassed in an ultrasonic bath before use. Prior to chromatographic analysis, the column was conditioned with the mobile phase for 30 min at a flow-rate of 1.3 ml/min.

At the beginning of the analysis, with the valve in load position (Fig. 1A), 500 μl of water were injected into the Guard-Pak C_{18} cartridge through the needle port by using a 500-μl Model 750 Hamilton syringe. Then, a sample aliquot, usually 0.1–0.5 ml (volumes up to 2 ml may be loaded), was injected and the C_{18} cartridge was washed with 500 μl of water by means of the syringe; finally, the valve handle was rotated to the inject position (Fig. 1B) to bring the eluting phase from the pump to the opposite end of the Guard-Pak module and to determine the sample to be flushed into analytical column. The chromatographic analysis was carried out isocratically at 1.3 ml/min at room temperature.

Peak identification

The 5-HT peak was identified on the basis of liquid chromatographic retention behaviour and by co-injection with the reference compound.

Quantitative analysis

The endogenous concentration of 5-HT in PPP and PRP was assessed by the

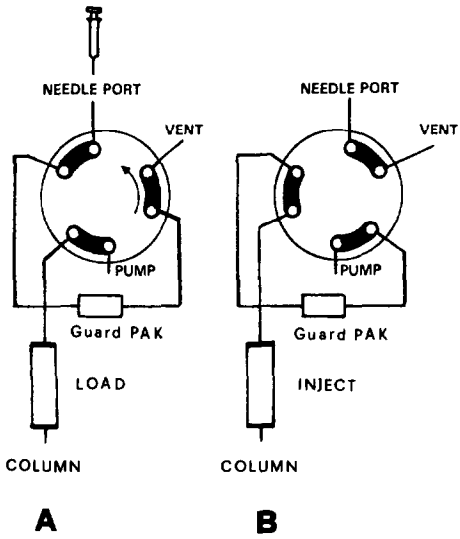

Fig. 1. Scheme for pre-column sample enrichment. With the valve in LOAD position (A), the sample is injected by a syringe on to the Guard-Pak module, which retains the compound of interest. Undesired compounds that are not adsorbed are flushed to waste by washing the Guard-Pak cartridge with water. The adsorbed compounds are then flushed from the cartridge to the analytical column by switching the valve to the INJECT position (B).

internal standard method. Peak-area ratios of 5-HT and I.S. in sample and standard were calculated, and the 5-HT concentration was determined according to the following equations:

$$\text{5-HT (PPP, ng/ml)} = \frac{R_s}{R_{st}} \times C_{st} \times D \quad (1)$$

$$\text{5-HT (PRP, ng per } 10^8 \text{ platelets)} = \frac{R_s}{R_{st}} \times C_{st} \times \frac{D}{N} \quad (2)$$

where R_s is the ratio of 5-HT peak area to that of I.S. standard for sample, R_{st} is the ratio of 5-HT peak area to that of I.S. for the standard, C_{st} is standard concentration in ng/ml, D is the dilution factor and N is 10^{-8}/ml platelet number.

RESULTS AND DISCUSSION

Our observations (unpublished) have indicated that 5-HT, as well as other indolic compounds (5-hydroxyindoleacetic acid, tryptophan) in diluted perchloric or trichloroacetic acid solution are quantitatively adsorbed on a Sep-Pak C_{18} cartridge. This has allowed us to devise an efficient and simple chromatographic system for pre-concentration and on-line purification of sample on a Guard-Pak C_{18} module. The analytical determination can then be carried out on an ion-exchange column, on-line with the pre-concentration cartridge. Optimization of the working conditions leads to the success of the analytical procedure. Thus, the final 3% trichloroacetic acid concentration used in the extraction step

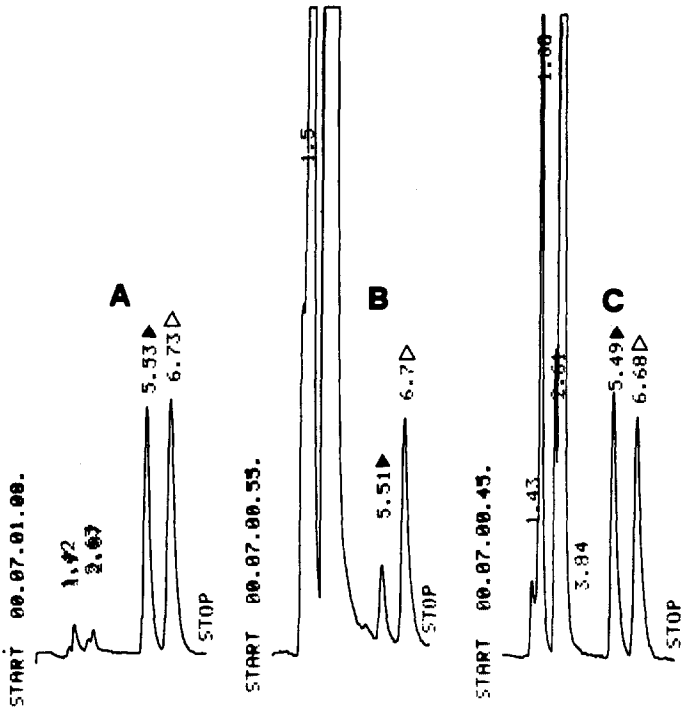

Fig. 2. (A) Chromatogram of a standard mixture of 1 ng each of 5-HT and I.S., in 0.5 ml of 3% trichloroacetic acid solution. (B) Chromatogram of a 0.5-ml acid extract of PPP sample. (C) Chromatogram of a 0.1-ml acid extract of PRP sample. Chromatographic conditions are described in the text. Peaks: ▲ = 5-HT; △ = I.S.

ensures complete protein precipitation, without appreciable degradation of the amine or of the siliceous matrix of C_{18} phase. The concentration of acetonitrile used in the mobile phase allows complete desorption within the void volume of the pre-concentration cartridge, without causing interference in the analytical separation on the ion-exchange column. The ionic modifier concentration and the mobile phase pH are set for isocratic elution and in a short analysis time.

Fig. 2A shows a typical chromatogram of 5-HT and N-methylserotonin: 1 ng of each were injected in 0.5 ml of 3% trichloroacetic acid solution. As can be seen, 5-HT is baseline-resolved from I.S. in less than 8 min.

The fluorimetric detector response increased linearly with 5-HT concentration up to 50 ng of amine injected and the correlation coefficient was 0.9989. If a signal-to-noise ratio of 4 is assumed, the detection limit of the assay is 200 pg of injected indoleamine, which is far below the concentration levels of plasma specimens.

Typical chromatograms of the analyses of PPP and PRP samples are shown in Fig. 2B and C, respectively. The analytes of interest are well separated from the other endogenous plasma compounds adsorbed on to the C_{18} cartridge. 5-Hydroxyindoleacetic acid and tryptophan are poorly retained by the exchanger and are eluted at retention times lower than those of 5-HT and I.S.

The reproducibility of the method, evaluated from multiple analyses of PRP

or pooled PPP samples, is quite satisfactory. The within-day coefficients of variation (C.V.), determined from five analyses of PRP and PPP, were 3.2 and 4.2%, respectively. The between-day C.V. determined from five analyses of the same samples, were 4.6% for PRP and 5.1% for PPP.

Recovery experiments, carried out by adding known amounts of serotonin and I.S. to PPP samples, gave values of $91 \pm 2.3\%$ and $90 \pm 3.1\%$, respectively (mean $\pm$ S.D. of five determinations).

The concentration of 5-HT in PPP samples of healthy male adults was 2.1 ± 0.8 ng/ml, while that of PRP serotonin was $65 \pm 18/10^8$ platelets. Values obtained in this work for PPP and PRP serotonin are in agreement with those obtained by using closely related techniques for sample preparation [13,17].

CONCLUSIONS

Analysis of 5-HT in plasma requires a sample clean-up step prior to final analysis by HPLC. Due to sample dilution during the purification step, the use of gravity-fed isolation columns can be somewhat troublesome, especially when the amine levels are low, as in the case of PRP. The use of trace enrichment for on-line pre-concentration of the amine in the purified sample may be a successful approach to sample dilution. Currently, this is accomplished by using a chromatographic system that involves a pump, an injection valve and a short column, as a pre-concentration unit, as well as the analytical chromatographic module [19].

In our work, pre-concentration and purification of the sample is performed by using a simplified device, which requires only a Guard-Pak module inserted on-line in the injection valve; cartridge loading and washing are carried out by means of a syringe. This system, coupled to an ion-exchange analytical column, allows the rapid, simple HPLC determination of 5-HT directly on acid extracts of plasma samples.

The pre-column concentration system described here can be used in HPLC as a suitable tool for on-line sample pre-concentration.

REFERENCES

1 E. Garelis, Adv. Exp. Med. Biol., 133 (1981) 717.
2 G.A. Kennet and M.H. Joseph, Neuropharmacology, 20 (1981) 39.
3 J. Barchas and E. Usdin (Editors), Serotonin and Behavior, Academic Press, New York, 1973.
4 D.G. Grahame-Smith, in S.C. Trielove and E. Lee (Editors), Topics in Gastroenterology, Blackwell, London, 1977, p. 285.
5 P.M. Vanhoutte, in F. De Clerke and P.M. Vanhoutte (Editors), 5-Hydroxytryptamine in Peripheral Reactions, Raven Press, New York, 1982, p. 163.
6 P. Frattini, M.L. Cucchi, G. Santogostino and G.L. Corona, Clin. Chim. Acta, 92 (1979) 353.
7 A. Parbtani and J.S. Cameron, Thromb. Res., 15 (1979) 109.
8 D.R. Shuttelworth and J.O. Rien, Blood, 57 (1981) 505.
9 G.T. Vatassery, M.A. Sheridan and A.M. Krezowski, Clin. Chem., 27 (1981) 328.
10 J.M. Kellum and B.M. Jaffe, Gastroenterology, 70 (1976) 516.
11 F. Engbaek and B. Voldy, Clin. Chem., 28 (1982) 624.
12 M.N. Hussain and M.J. Sole, Anal. Biochem., 111 (1981) 105.

13 D.D. Koch and P.T. Kissinger, Anal. Chem., 52 (1980) 27.
14 W.H. Lyness, N.M. Friedle and K.E. Moore, Life Sci., 26 (1980) 1109.
15 P.C. Tagari, D.J. Boulin and C.L. Davies, Clin. Chem., 30 (1984) 131.
16 W. Buczko, R. Invernizzi and G. de Gaetano, Thromb. Haemostas., 51 (1984) 131.
17 M. Picard, D. Olichon and J. Gombert, J. Chromatogr., 341 (1985) 445.
18 J. Jouve, J. Martineau, N. Mariotte, C. Bartelemy, J.P. Muh and G. Lelord, J. Chromatogr., 378 (1986) 437.
19 D.D. Koch and P.T. Kissinger, Life Sci., 26 (1980) 1099.