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DETERMINATION OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID IN PLASMA BY DIRECT INJECTION IN COUPLED-COLUMN LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method is described that allows determination of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in platelet-poor human blood plasma after direct injection of 50–100 μl of plasma into a coupled-column liquid chromatographic system. The chromatographic system comprised an enrichment column and two separation columns with different selectivity properties. The samples were injected into the C_{18} enrichment column, which was eluted with a buffer solution as the mobile phase. 5-HT, 5-HIAA and the internal standard 5-hydroxy-N-methyltryptamine (5-HMT) were then desorbed by a stronger mobile phase and flushed into a cation exchanger, which separated 5-HT and 5-HMT. 5-HIAA passed straight through and was switched to a C_{18} column where it was retarded. After 5-HT and 5-HMT had been eluted from the cation exchanger and detected, the eluate from the C_{18} column was directed to the detector and 5-HIAA was determined. Basic plasma levels, about 4 n*M* for 5-HT and 30 n*M* for 5-HIAA, were measured with a relative standard deviation of about 5%.

INTRODUCTION

Blood platelets are a primary source of 5-hydroxytryptamine (5-HT), and measurements of intra- and extraplatelet concentrations of this biogenic amine are of interest in studies of haemostasis, thrombosis and different cardiovascular diseases[1,2].

A very sensitive method is needed for assaying platelet-poor plasma samples, where the concentration of 5-HT can be of the order of a few picomoles per ml. Various methods have been applied to the determination of 5-HT in biological fluids. Liquid chromatography with fluorometric[3,4] or electrochemical[5–8] detection provides high sensitivity. The assays often require precipitation of proteins[3,4] or sample purification and concentration steps, such as solvent extraction[6–8] or ion-exchange extraction[5]. Several authors have reported lowered recoveries after precipitation of proteins, probably due to co-precipitation of the compounds or decreased stability in the acidic environment[4,9–11].

We have developed a method for the concurrent measurement of 5-HT and its

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metabolite 5-hydroxyindoleacetic acid (5-HIAA) based on liquid chromatography with coupled column and electrochemical detection. The method is rapid and sensitive and does not require pre-purification of the plasma sample.

EXPERIMENTAL

Apparatus

The liquid chromatograph comprised three pumps, Model 2150 (LKB-Produkter, Bromma, Sweden), a pulse dampener (Touzart-Matignon, Vitry-sur-Seine, France), an automatic injector, ISS-100 (Perkin-Elmer, Überlingen, F.R.G.), with a refrigerated sample tray and injection vials (0.3 ml) of borosilicate glass (Chromacol, London, U.K), a Model 4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.) and a M 460 electrochemical detector (Waters Assoc., Milford, MA, U.S.A.), in combination with a TL-5A thin-layer cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.), comprising a glassy carbon working electrode, a stainless-steel auxiliary electrode and a silver–silver chloride reference electrode.

The switching events were controlled by the integrator and were performed by three pneumatically operated six-port valves, Model 7010 (Rheodyne, Berkeley, CA, U.S.A.) and an interface constructed by the Instrument Department, Hässle (Mölndal, Sweden). A refrigerated microcentrifuge, Model 154 RF (Ole Dich, Instrumentmakers, Hvidovre, Denmark), was used for centrifugation and a MSE (Crawley, U.K.) Soniprep 150 ultrasonic disintegrator for the disruption of platelets.

Chemicals

5-HT hydrochloride, 5-HIAA, 5-hydroxyindole-2-carboxylic acid (5-HICA), N-acetyl-5-hydroxytryptamine (Ac-5-HT), ethylene glycol bis (β-aminoethyl ether)-N′,N′-tetraacetic acid (EGTA) and reduced glutathione (GSH) were obtained from Sigma (St. Louis, MO, U.S.A.), 5-hydroxy-N-methyltryptamine (5-HMT) oxalate from EGA-Chemie (Steinheim, F.R.G.), 5-hydroxyindole-3-propionic acid (5-HIPA) and 5-hydroxytryptophol (5-HTOL) from Regis (Morton Grove, IL, U.S.A.) and hexanesulphonic acid sodium salt from Eastman Kodak (Rochester, NY, U.S.A.). Disodium ethylenediaminetetraacetate (EDTA), citric acid and all buffer substances and inorganic acids were of analytical reagent grade (E. Merck, Darmstadt, F.R.G.), and acetonitrile and methanol were of HPLC grade (Rathburn, Walkerburn, U.K.).

Chromatography

A schematic drawing of the chromatographic system is shown in Fig. 1, and a time schedule for the switching of the valves is presented in Table I. The enrichment column was a LiChroprep RP-18 column (50 mm × 4.6 mm I.D.) with 15–25 or 25–40 μm particles from E. Merck and 2-μm titanium frits from Upchurch (Oak Harbor, WA, U.S.A.). The separation columns were a Nucleosil 5 C_{18} column (150 mm × 4.6 mm I.D.) and a cation-exchange column, Nucleosil 5SA (50 mm × 3 mm I.D.), packed with particles from Macherey-Nagel (Düren, F.R.G.).

The enrichment and cation-exchange columns were packed with a viscosity slurry packing technique using methanol as the pressure liquid and a suspending liquid consisting of toluene–dioxane–cyclohexanol (1:1:4) and toluene–cyclohexanol (4:5), respectively[12]. The C_{18} column was obtained prepacked.

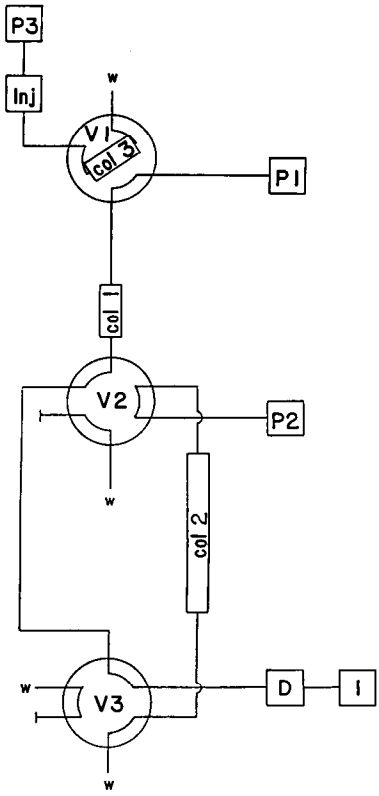

Fig. 1. Coupled-column liquid chromatographic system. P1, P2 and P3 = pumps with mobile phases 1 (citrate buffer, pH 5, $I=0.2$, containing 13% methanol), 2 (citrate buffer, pH 4.5, $I=0.2$, containing 10% methanol) and 3 (citrate buffer, pH 4, $I=0.1$); Inj = autoinjector; Col 1 = Nucleosil 5SA; Col 2 = Nucleosil $5C_{18}$; Col 3 = LiChroprep RP-18; V1, V2 and V3 = six-port valves; D = electrochemical detector; I = integrator; w = waste. For further details, see text.

TABLE I
TIME SCHEDULE FOR THE COLUMN SWITCHING

Time after injection (min)	*Switch valve No.*	*Comment*
0.0		The sample is injected into the enrichment column (3) with buffer for 3 min
3.0	1	The indoles are back-flushed from column to the cation-exchange column (1) with buffer containing methanol
3.6	2	5-HIAA is switched from column 1 to the C_{18} column (2) for 1.3 min
4.9	2 reset	The eluate from column 1 is recorded
14.0	3	The eluate from column 2 is recorded
14.1	1 reset	Column 3 is equilibrated with buffer
19.0	3 reset	The chromatogram is completed, and after recording the results, the next sample is injected

The mobile phases were citrate buffer (pH 4; ionic strength, $I=0.1$) for the enrichment column, citrate buffer (pH 5, $I=0.2$) containing 13% methanol for the separation on the cation exchanger and citrate buffer (pH 4.5, $I=0.2$) containing 10% methanol for the separation on the C_{18} column. The compositions of the buffers were 70 m*M* citric acid and 82 m*M* sodium hydroxide for pH 4, $I=0.1$, 70 m*M* citric acid and 131 m*M* sodium hydroxide for pH 5, $I=0.2$ and 95 m*M* citric acid and 145 m*M* sodium hydroxide for pH 4.5, $I=0.2$. Deionized and filtered water (Milli Q; Millipore, Bedford, MA, U.S.A.) was used for the mobile phases, which, prior to use, were degassed and filtered through a 0.45-μm MF Millipore filter. The flow-rate was 1 ml/min at ambient temperature and the eluent was monitored with an electrochemical detector, operated at +0.6 V. The detector cell was housed in a Faraday cage to avoid electrical disturbances.

Sample preparation

The blood samples were collected in chilled tubes of polypropylene, containing a small volume (20 μl/ml blood) of a solution (pH 5–7) of an anticoagulant, EGTA (0.2 *M*), and an antioxidant, GSH (0.2 *M*). These stabilizing agents are commonly used for catecholamines. After gentle mixing, the contents of the tubes were centrifuged at 10 000 *g* and 4°C for 10 min. The samples were stored at −70°C until analysed. After thawing and mixing of the sample, 100 μl of plasma and 20 μl of the internal standard, 5-HMT (2 μ*M*), were added to the injection vial which was capped and vortex-mixed before injection of 10–100 μl into the chromatographic system. Standard solutions of 5-HT, 5-HIAA and 5-HMT were prepared in phosphate buffer (pH 6), containing 5 m*M* EDTA, and when kept in portions at −70°C they were stable for 1 year. The buffer composition was 69 m*M* sodium dihydrogenphosphate and 10 m*M* disodium hydrogenphosphate.

Calibration was performed by injection of a mixture of 100 μl standard solution and 20 μl internal standard solution, and by measuring peak-height ratios. The median value of the calibration samples were used to calculate the concentrations of 5-HT and 5-HIAA in the unknown samples.

RESULTS AND DISCUSSION

Chromatography

Resolution of 5-HT, 5-HIAA and the internal standard, 5-HMT, was easily accomplished on a Nucleosil $5C_{18}$ column (150 mm × 4.6 mm I.D.) with a mobile phase of citrate buffer (pH 5, $I=0.1$) with 10% methanol as the organic modifier. Rabbit plasma samples containing *ca.* 100 n*M* of 5-HT and 5-HIAA were analyzed after direct injection of 10 μl of plasma. However, when an higher sensitivity was required, 5-HT was incompletely separated from interfering components originating from the plasma matrix. An increase in the retardation of 5-HT, by adding hexanesulphonate as counter ion to the mobile phase, did not adequately improve the selectivity.

Two-column system

By replacing the C_{18} column with a cation-exchange column, Nucleosil 5SA (50

mm × 3 mm I.D.), clean chromatograms of 5-HT were obtained while 5-HIAA was eluted too close to the front peak. This problem was solved by use of the cation-exchange column and the C_{18} column with a column-switching valve. After passage through the cation exchanger, 1.3 ml of the eluate, containing 5-HIAA, were switched to the C_{18} column, where 5-HIAA was retarded. The mobile phase for the cation exchanger was a citrate buffer (pH 5.0, $I=0.2$) containing 13% methanol.

After 5-HT and 5-HMT had been eluted from the cation exchanger and detected, the eluate from the C_{18} column was directed to the detector by a second valve and 5-HIAA was determined. To obtain a suitable retention time of 5-HIAA on the C_{18} column, a mobile phase, containing 10% methanol, an ionic strength of 0.2 and a pH of 4.5, was used.

This chromatographic system comprising two separation columns, a cation-exchange and a C_{18} column, was used for direct injection of rabbit plasma samples when 10 μl or less were sufficient to enable the measurement of 5-HT and 5-HIAA. A guard column (15 mm × 3 mm I.D.), containing 3-μm CN particles (Brownlee, Santa Clara, CA, U.S.A.), was inserted before the cation-exchange column and replaced when a total volume of about 400 μl of plasma had been injected into it. The cation exchanger was replaced when retention times became shorter, after assaying *ca.* 250 plasma samples. In Fig. 2 is shown a chromatogram obtained after injection of 10 μl of rabbit plasma into the two-column system.

We also performed experiments in which the cation exchanger was used in combinaton with an anion exchanger, Spherisorb SAX, 5-μm, 150 mm × 4.6 mm I.D. (PhaseSep, Norwalk, CT, U.S.A.). 5-HIAA was retarded on the anion exchanger, with

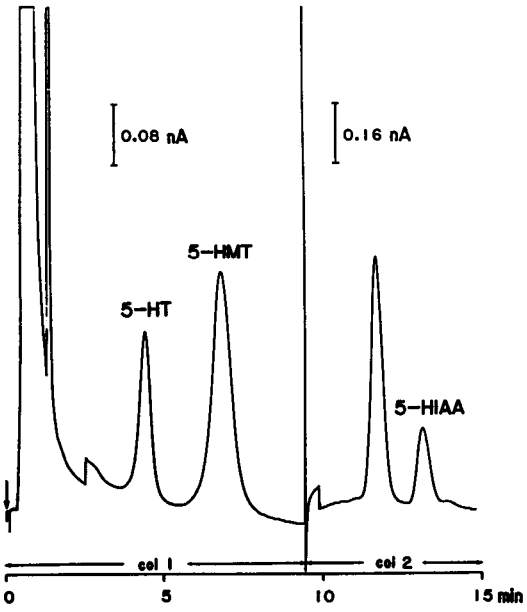

Fig. 2. Chromatogram of a rabbit plasma sample, containing 377 n*M* of 5-HT, 995 n*M* of 5-HMT and 354 n*M* of 5-HIAA, injected into the two-column liquid chromatographic system. Injection volume: 10 μl.

pure citrate buffer as the mobile phase, while 5-HT passed straight through and was switched to the cation exchanger. However, when plasma samples were injected directly into the anion-exchange column, we did not succeed in obtaining clean chromatograms of 5-HIAA.

Three-column system

For platelet-poor human plasma samples with very low concentration levels of 5-HT, injection volumes of 50–100 μl were required. To enable direct injection of these quantities of plasma, an enrichment column was placed in the loop position of a valve, and the plasma samples were applied to this column with a buffer solution as the mobile phase. After an appropriate time, 5-HT, 5-HIAA and 5-HMT were desorbed and backflushed with the stronger eluent into the cation exchanger, and the separations were performed as described above for the two-column system. While 5-HIAA was being measured, the buffer solution was switched back to the enrichment column to equilibrate it before the next injection. In Fig. 3 is shown a chromatogram obtained after injection of 100 μl of an human plasma sample into the three-column system.

Enrichment column

Precolumns with various packing materials were tested for their retardation ability and column efficiency. This was accomplished by connecting the detector directly to the effluent of the enrichment column. Columns containing packing materials with 5-μm particle diameters, *e.g.*, prepacked C_{18} columns (30 mm × 4.6 mm I.D.) from Brownlee gave too high a back pressure after injection of a total volume of a few hundred microlitres of plasma, and C_{18} columns (40 mm × 4.6 mm I.D.) containing particles of diameter 10 and 40 μm did not show any retardation of the

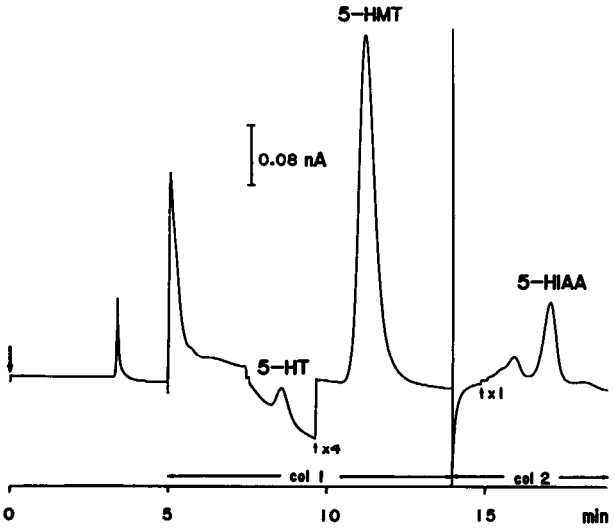

Fig. 3. Chromatogram of an human plasma sample, containing 3.8 n*M* of 5-HT, 238 n*M* of 5-HMT and 26.2 n*M* of 5-HIAA, injected into the three-column liquid chromatographic system. Injection volume: 100 μl.

solutes. We then packed columns (50 mm × 4.6 mm I.D.), equipped with titanium frits instead of steel frits, with various packing materials. According to reports in the literature, steel frits may interact with proteins, causing blockage and thus higher back pressure[13,14]. Another advantage of titanium frits, over steel frits, is that they gave a lower background current in electrochemical detection and can be used without any pretreatment. Steel frits had to be treated with a strong acid in an ultrasonic bath before use in order to lower the background current.

The packing materials LiChroprep RP-18, 15–25 and 25–40 μm, gave satisfactory results. A retention time of 9.5 min for 5-HT, the least retarded compound, was obtained with citrate buffer ($I=0.1$, pH 4) as the mobile phase. The buffer was passed through the column for 3 min, to wash out rapidly eluted compounds, before desorbing the solutes by backflushing with the stronger eluent. With citrate buffer as the mobile phase, the retardation of 5-HIAA reached a maximum at pH of *ca.* 4. At more acidic pH, less retardation was unexpectedly obtained. This might be due to displacement effects. A total plasma volume of about 5 ml was injected into the column before replacement.

Internal standard

An internal standard was added to compensate for variations in the injection volume and for minor fluctuations in the detector response during the analysis. Both acidic and basic substances were tested as internal standards. The degrees of protonation of the compounds and, hence, their retention times were altered by changing the pH of the mobile phase. The influence of pH on the capacity factors (k') is illustrated in Fig. 4. For the acidic substances, on the C_{18} column, the k' values decreased, not only when the pH of the mobile phase increased and the substances were

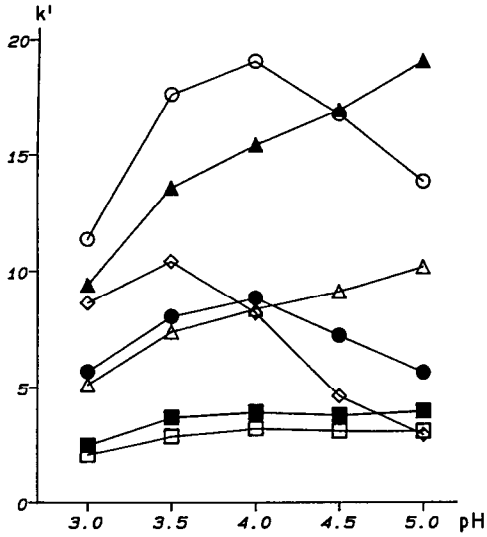

Fig. 4. Influence of the pH of the mobile phase on retention. Stationary phase: Nucleosil $5C_{18}$. Mobile phase: citrate buffer ($I=0.2$) containing 10% methanol. Key: □ = 5-HT; ■ = 5-HMT; △ = 5-HTOL; ● = 5-HIAA; ◇ = 5-HICA; ▲ = Ac-5-HT; ○ = 5-HIPA.

charged, but also, as mentioned above, at the lower pH of the citrate buffer. A maximum retardation was shown at a pH of about 3.5 for 5-HICA and at pH 4 for 5-HIAA and 5-HIPA. With 5-HICA as the internal standard, a pH of *ca.* 3 was suitable. However, at this pH the chromatogram of a plasma sample showed interferences with the 5-HIAA peak. Of the basic substances tested, 5-HMT was well separated from 5-HT on the cation exchanger and was therefore chosen as the internal standard.

Stability

5-HIAA was not stable in acidic solutions, *e.g.*, 0.01–0.1 *M* hydrochloric or perchloric acid, as also reported by others[15,16]; this is why we further examined the stability of 5-HT, 5-HMT and 5-HIAA in phosphate buffer at pH 6. Addition of EDTA (5 m*M*) improved the stability of the indole derivatives, their solutions being stable for at least 1 month when stored, in a dark place, at room temperature. At +4°C the solutions were stable, during this period, even in the absence of EDTA and at −70°C the solutions containing EDTA (5 m*M*) were stable for more than 1 year.

Accuracy

Precautions must be taken to prevent platelet breakdown during blood sampling, as 5-HT is mainly located within the platelets, and disrupted platelets will give rise to falsely increased plasma levels[4,17]. Fig. 5 shows that a centrifugation speed

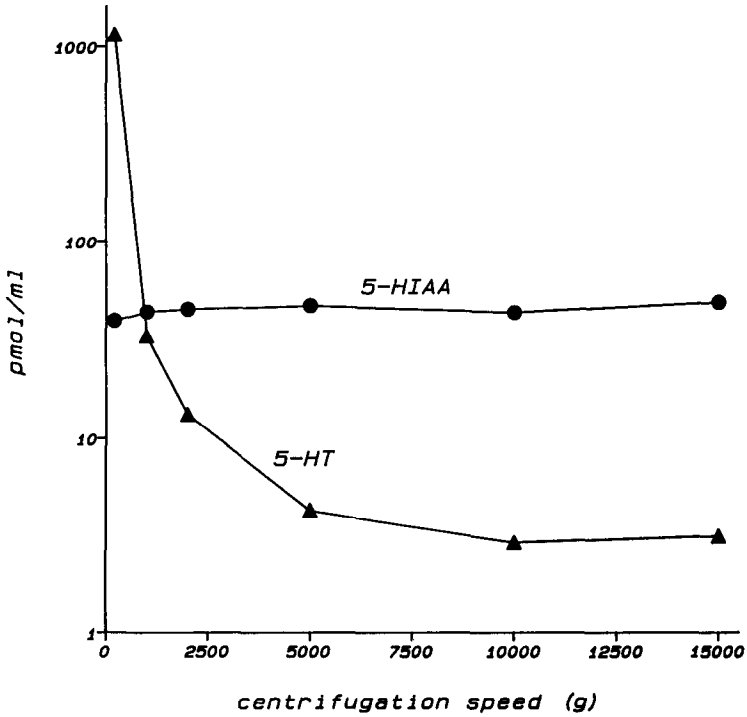

Fig. 5. Effect of centrifugation speed on the concentration of 5-HT in a plasma sample.

of 10 000 *g* was needed to obtain complete sedimentation of platelets. Centrifugation at 15 000 *g* gave the same result as centrifugation at 10 000 *g*, showing that no disruption of platelets occurred during centrifugation. This observation differs from that of Picard *et al.*[7], who found an higher concentration of 5-HT after centrifugation at 13 000 *g* than at 6000 *g*. 5-HIAA was not affected by the centrifugation speed, indicating that it is mainly located outside the platelets.

When plasma samples, containing intact platelets, were analyzed they were first treated with ultrasound or frozen at −70°C overnight to disrupt the platelets, otherwise most of the platelets adhered to the enrichment column, retaining their content of 5-HT. This gave falsely low values of the 5-HT concentration and caused contamination of subsequent chromatograms.

Many assays involve precipitation of proteins, but this leads to losses which must be compensated for in the calculation of the recovery[4,9–11]. After precipitation of proteins with perchloric acid, we observed losses of 5 to 10% for 5-HT and 5-HMT and *ca.* 30% for 5-HIAA.

Recovery and standard deviation

The recoveries of the indoles were determined by injecting standard solutions of the amines and the acid directly into the cation-exchange and reversed-phase column, respectively. The peak areas were compared with those obtained after injection into the coupled-column system. The recoveries were found to be 99.2 ± 1.2% for 5-HT, 97.3 ± 1.4% for 5-HMT and 101.6 ± 2.4% for 5-HIAA ($n=5$). Recoveries obtained when plasma samples were injected into the chromatographic system were 100.7 ± 2.6% and 98.2 ± 1.9% for 5-HT and 5-HIAA, respectively ($n=4$). For the concentration range tested (1–5000 n*M*) linearity was found for both 5-HT and 5-HIAA, in aqueous and plasma samples. The injection volume was 50 μl.

The relative standard deviations for injection of 10 μl of rabbit plasma into the two-column system were 2.4% for 5-HT and 3.9% for 5-HIAA ($n=10$) at a concentration of 380 n*M* of 5-HT and 270 n*M* of 5-HIAA. For a standard sample, the corresponding values were 1.1 and 1.6% at a concentration of 1.5 μ*M*. For a standard sample (100 μl), injected into the three-column system, the relative standard deviations were 3.4 and 3.0% for 5-HT and 5-HIAA, respectively ($n=5$), at a concentration of 26.1 n*M* for 5-HT and 36.3 n*M* for 5-HIAA. The corresponding values for an human plasma sample were 3.5% for 5-HT and 5.7% for 5-HIAA ($n=10$) at concentrations of 3.8 and 26.2 n*M*, respectively. The limit of detection was estimated as 1 n*M*.

The mean levels of 5-HT and 5-HIAA in platelet-poor plasma samples, obtained from normal controls ($n=5$), were 3.73 ± 0.65 and 30.5 ± 19.2 n*M*, respectively, in agreement with published results[4,11,18].

The concentrations of the plasma samples were calculated by comparing the peak-height ratios of 5-HT or 5-HIAA and the internal standard in the plasma samples with the ratios in the standard samples, which were prepared from aqueous standard solutions with known concentrations.

CONCLUSION

The chromatographic method described here permits quantification of 5-HT and 5-HIAA in less than 100 μl of a platelet-poor plasma sample. It is rapid and

relatively simple, requiring no pretreatment of the sample except for addition of the internal standard before injection. A recovery of about 100% was obtained, and the relative standard deviations were 3.5 and 5.7% for 5-HT and 5-HIAA, respectively, when an human plasma sample, containing 3.8 n*M* 5-HT and 26.2 n*M* 5-HIAA was analyzed.

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