Journal of Chromatography, 419 (1987) 85–94
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3696

DETERMINATION OF SEROTONIN AND 5-HYDROXYINDOLEACETIC ACID IN URINE BY REVERSED-PHASE ION-PAIR PARTITION CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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(First received December 23rd, 1986; revised manuscript received March 6th, 1987)

SUMMARY

The determination of serotonin and 5-hydroxyindoleacetic acid in urine is described. The compounds are separated by means of reversed-phase chromatography using tri-*n*-butylphosphate as the stationary phase and a mobile phase containing perchlorate as an ion-pairing agent. Detection is performed fluorimetrically. The compounds can be assayed directly, using two mobile phases with different pH values. Alternatively, both compounds can be determined with the same mobile phase after urine extraction on a small XAD-2 column for clean-up of serotonin. Two different approaches were evaluated for this XAD-2 extraction, viz.: (i) desorption of serotonin with a methanol-rich solvent after a washing step with an aqueous solvent; (ii) direct desorption of serotonin in the washing step using a solvent with a small percentage of methanol. Only the latter approach proved to be successful. The linearity of the method was investigated by standard additions to urine. The method was applied to the determination of both compounds in urine. The stability of the compounds in standard solutions and in urine was investigated under various storage conditions.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is mainly stored in blood platelets and in the enterochromaffin cells of the gastrointestinal tract. Carcinoid tumours, mainly originating from the gastrointestinal tract, are characterized by an excessive production of 5-HT and can be diagnosed by increased levels of 5-HT and/or 5-hydroxyindoleacetic acid (5-HIAA) in urine [1]. The combined determination of 5-HT and 5-HIAA in urine could yield information on the histogenesis and metastatic spread of the carcinoid tumour.

Nowadays high-performance liquid chromatography (HPLC) is the most popular method for the determination of indole derivatives, such as 5-HT and 5-

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0378-4347/87/$03.50

HIAA, in body fluids and tissues. 5-HIAA has been assayed in urine after filtration and/or centrifugation [2–10], liquid–liquid extraction with ethyl acetate [11,12] or diethyl ether [13], liquid–solid extraction with Sephadex [14–17], C_{18} cartridges [18,19] or XAD-2 resin [20]. Sample pretreatment of urine for the determination of 5-HT was performed by centrifugation and filtration [8], liquid–solid extraction with XAD-2 resin [20] or cation-exchange material [16,21,22] or by ethyl acetate extraction after derivatization with *o*-phthalaldehyde [23]. The simultaneous assay of 5-HT and 5-HIAA from urine by HPLC has been described in only one paper [8]. However, for the separation a step gradient was required with analysis times of ca. 40 min, and no baseline separation of 5-HT and 5-HIAA was obtained.

Our purpose was to develop a method for the determination of 5-HT and 5-HIAA in urine, requiring only minimal sample pretreatment. We investigated the possibilities of direct injection of urine under various chromatographic conditions in order to determine both compounds simultaneously; furthermore, we evaluated sample pretreatment by means of liquid–solid extraction on small XAD-2 columns as described previously by Segura et al. [20]. Similar extraction procedures have been described, based on the adsorption of 5-HT and 5-HIAA through apolar interactions, and using C_{18} cartridges for the determination of indoles in urine [18,19] or TSK resin for the determination of indoles in plasma [25].

The chromatographic system that we used for the separation was modified from a reversed-phase ion-pair system developed for the separation of 5-HT and its precursors and metabolites [24]. This system, which uses tri-*n*-butylphosphate (TBP) as the liquid stationary phase, can be applied to the assay of 5-HT, 5-HIAA, 5-hydroxytryptophan, tryptophan and tryptamine in brain tissues [24]. Preliminary results showed that the system is also suited to the direct determination of 5-HIAA in urine samples [24], which application was also described by Wahlund and Edlén [4,5].

EXPERIMENTAL

Apparatus

The apparatus was essentially the same as described previously [24]. The compounds were separated on laboratory-packed stainless-steel columns (100×3.0 mm I.D.) and detected by means of their native fluorescence, using an SFM 23 LC spectrofluorimeter (Kontron, Zürich, Switzerland), with excitation and emission wavelengths set at 296 and 333 nm, respectively. Peak areas were calculated by means of a Chromatopac-E1A integrator (Shimadzu, Kyoto, Japan).

Chemicals and reagents

The chromatographic support material Polygosil C_{18} (mean particle size 5 μm) was obtained from Macherey & Nagel (Düren, F.R.G.). Serotonin creatinine sulphate monohydrate and 5-HIAA were obtained from Aldrich (Beerse, Belgium) as was TBP (99%). Water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical or reagent grade and were used without further purification.

Chromatography

The column-packing procedure and the technique used for the in situ loading of the column with TBP were as described previously [24]. Unless stated otherwise, the mobile phase consisted of water–methanol (85:15, v/v) containing 0.05 *M* oxalate and 0.15 *M* perchlorate. Prior to the addition of the methanol, the pH of the mobile phase was adjusted to 4.2. The mobile phase and column were thermostatted at 35 or 41 °C. In order to avoid stripping of the stationary phase, the mobile phase must be saturated with TBP, which is done by squirting TBP (ca. 2–5 ml/l) into the mobile phase, adjusted to the appropriate temperature. The mobile phase is ready for use when the two phases have separated, which takes ca. 15 min when the solution in the eluent reservoir is stirred gently.

Sample pretreatment

After collection, urine samples were stored in containers wrapped in tinfoil at 4 °C or, if the time between sampling and analysis was longer than a week, at −20 °C. All sample handling was performed in containers wrapped in tinfoil or under exclusion of strong light.

For the determination of 5-HIAA, urine was diluted 1:1 (v/v) with 0.6 *M* hydrochloric acid and filtered through a plug of Whatman GF/B filter by centrifugation, as described elsewhere [26]. A 50-μl aliquot of the filtrate was injected on to the HPLC column.

For the determination of 5-HT, 10 ml of urine were acidified with 0.3 ml of 12 *M* hydrochloric acid, yielding a pH of ca. 0.8; 1 ml was applied to an XAD-2 column (see below) and allowed to drain through under gravity. 5-HT was eluted with 3 ml of water–methanol (8:2, v/v) containing 0.2% (w/v) mercaptoethanol. A 50-μl aliquot of the eluate was injected on to the HPLC column.

Preparation of the XAD-2 extraction columns

Before use, XAD-2 (Serva, Heidelberg, F.R.G.; particle size 150–200 μm) was washed in methanol. After sedimentation for 10 min, the supernatant, containing the smaller particles, was decanted. This procedure was repeated twice, after which the XAD-2 was dried, and 200 mg of it were poured into a Pasteur pipette between two plugs of cotton wool moistened with methanol. The column material was conditioned by washing it twice with 1.5 ml of methanol and then twice with 1.5 ml of water. The solvents were allowed to drain through under gravity. In order to improve the packing of the XAD-2 particles in the column, during the washing step with water, air pressure (ca. 5 bar) was applied to the column for a moment, preventing the column from being blown dry. After urine extraction the XAD-2 column could be reconditioned by applying the same washing procedure.

Calibration and quantification

For preparation of the calibration curves standards were prepared as follows: aliquots of a stock solution (10 μg/ml 5-HT and 300 μg/ml 5-HIAA in 0.3 *M* hydrochloric acid) and 0.3 *M* hydrochloric acid were combined to final volumes of 0.25 ml. Then 10 ml of water–methanol (8:2, v/v) containing 0.2% (w/v) mercaptoethanol and 0.3 ml of 12 *M* hydrochloric acid were added, yielding cali-

bration standards with final concentrations of 47.4–237 ng/ml 5-HT and 1.42–7.11 μg/ml 5-HIAA. The stock solution and calibration standards were stored at 4°C in containers wrapped in tinfoil (polypropylene for the standards, polypropylene or glass for the stock solution). Under these conditions, the stock solution was stable for one month; the standards should be prepared freshly every week. Quantification was performed by comparing the peak areas of the processed urine samples with the peak areas of the calibration curves.

Stability studies

The standards of pH 0.7 were prepared in 0.3 *M* hydrochloric acid, and from these the standards of pH 2.0 were prepared by addition of 5 *M* sodium hydroxide. The urine sample (pH 6.5) was acidified with 1 *M* hydrochloric acid to a final pH of 2.0 or with 12 *M* hydrochloric acid (0.3 ml plus 10 ml of urine) to a final pH of 0.8. Prior to HPLC injection the concentrated standards were diluted 200-fold with 0.3 *M* hydrochloric acid, whereas the diluted standards were injected directly. The urine samples were pretreated as described above, except for the urine of pH 0.8, which was applied directly on to the XAD-2 column for the determination of 5-HT or diluted 1:1 (v/v) with 0.3 *M* hydrochloric acid for the determination of 5-HIAA.

RESULTS AND DISCUSSION

Direct determinations in urine

Fig. 1 shows the effect of variation of the pH of the mobile phase on the selectivity of the chromatographic system. The retention of 5-HT hardly changes while the retention of the interfering compounds strongly depends on the pH, presumably as the result of acid–base equilibria. Thus, at pH 7.2, a rather clean chromatogram is obtained from which 5-HT can readily be quantified. However, at this pH the retention of 5-HIAA is negligibly small [24]. Consequently, the direct determination of 5-HT and 5-HIAA is only possible when two different mobile phases are used.

In addition to fluorimetric detection (see Fig. 1) amperometric detection was also used under conditions similar to those described elsewhere [24] at a working electrode potential of +0.8 V vs. Ag/AgCl. In our experience fluorimetric detection was more selective, so because the detection limits of both methods were about the same [24] only fluorimetric detection was used in further experiments.

Sample pretreatment on XAD-2

From the published methods a liquid–solid extraction on a non-polar adsorbent [18,20,25] seemed promising. In this method, which can be performed batchwise [20] or on small columns [18,25], interfering compounds are removed in a washing step and the indoles are desorbed with an organic solvent. The method is relatively simple, it can be used for 5-HT and 5-HIAA, and it allows the concentration of the compounds to be determined. We selected XAD-2, a copolymer of styrene and divinylbenzene, because it is cheap and easy to handle and it can be reconditioned and used repeatedly.

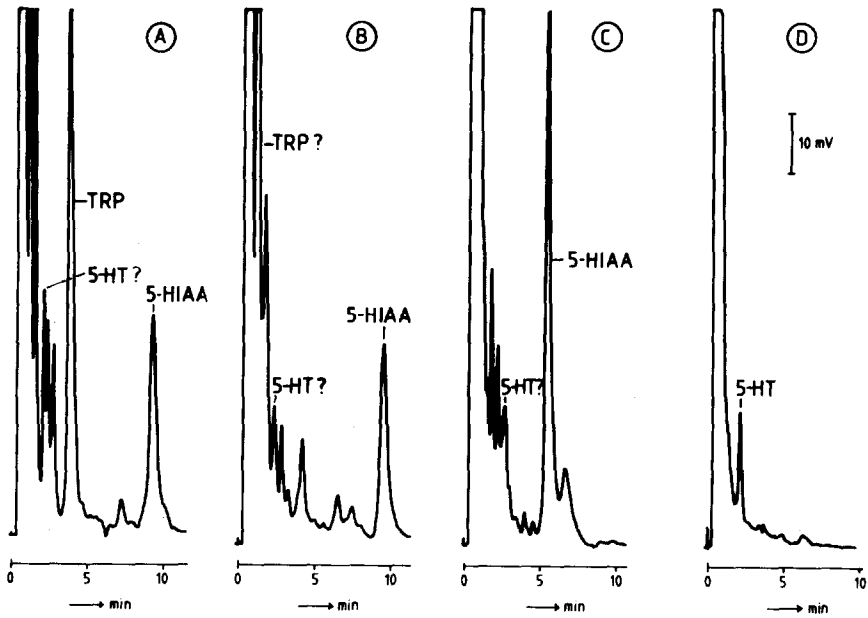

Fig. 1. Direct injection of urine: effect of the mobile phase pH on the selectivity of the chromatographic system. Mobile phase, water–methanol (8:2, v/v) with 0.15 *M* perchlorate and 0.05 *M* phosphate; pH of the aqueous part of the mobile phase, 2.2 (A), 3.5 (B), 4.8 (C) and 7.2 (D); temperature, 35 °C; flow-rate, 1.0 ml/min; injection volume, 40 µl. Peaks: TRP = tryptophan; 5-HT = serotonin; 5-HIAA = 5-hydroxyindoleacetic acid.

5-HIAA is significantly retained on XAD-2. At pH values of the urine sample and the aqueous washing solvent below 4, only a small fraction (less than 10%) is lost when the washing volume is 6 ml. In contrast, 5-HT has a low retention on XAD-2, even when an ion-pairing agent (0.3 *M* perchlorate) is added to the sample and to the washing solvent. Using a washing volume of only 2 ml, at least 10% of the 5-HT is lost in the washing step. Segura et al. [20], who used a batch extraction method, also found a low recovery (20%) of 5-HT on XAD-2. Nevertheless, they used this method for the determination of 5-HT in urine by means of gas chromatography–mass spectrometry. In our hands, the XAD-2 extraction did not lead to the desired clean-up: the compounds interfering in the determination of 5-HT were still present in the samples.

Because the above approach proved to be unsuccessful, we tried to find conditions under which 5-HT and 5-HIAA would be selectively eluted from the XAD-2 column while the interfering compounds are still retained. Recently, this approach was applied to the assay of 5-HIAA in urine, using C_{18} cartridges for sample clean-up [19].

Using water containing 0.3 *M* hydrochloric acid, the XAD-2 column must be washed with at least 6 ml for quantitative recovery of 5-HT and with a larger volume for quantitative recovery of 5-HIAA. The elution strength can be increased by addition of methanol: using an eluent that contains 20% (v/v) methanol, 5-HT is quantitatively recovered in 3 ml. This method of sample preparation results

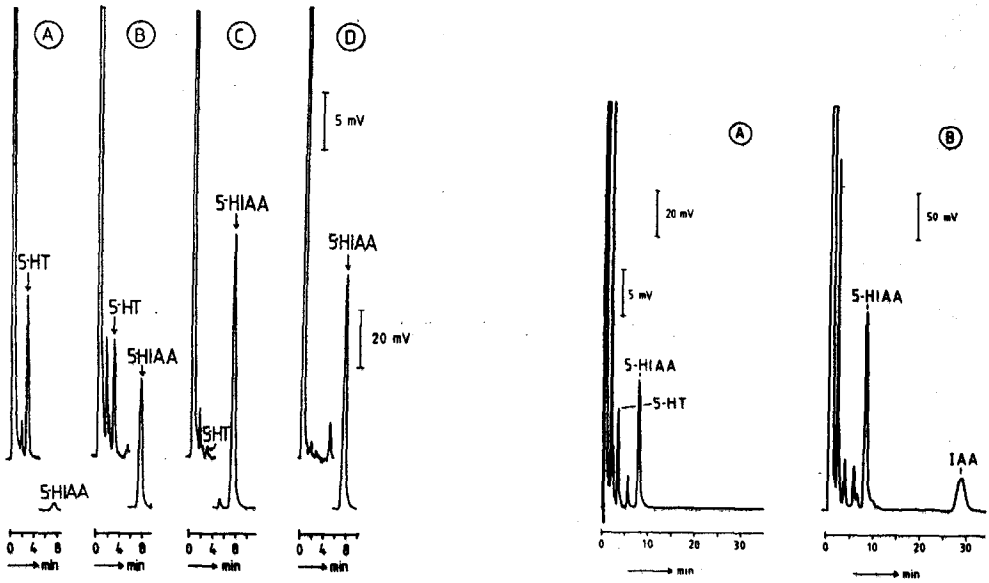

Fig. 2. Urine extraction on XAD-2: distribution of 5-HT in the first (A), second (B), third (C) and fourth (D) millilitre of the eluate of the XAD-2 extraction. Sample pretreatment, see Experimental (procedure for 5-HT); chromatographic system, see Experimental; temperature, 41 °C; flow-rate, 1 ml/min; injection volume, 50 µl.

Fig. 3. Determination in urine of (A) 5-HT after extraction on XAD-2; (B) 5-HIAA after 1:1 (v/v) dilution and filtration. Sample pretreatment procedures, see Experimental; chromatographic conditions, see Fig. 2; urine concentration, 94 ng/ml 5-HT and 5.2 µg/ml 5-HIAA. IAA = indoleacetic acid.

in a sufficient clean-up of the urine samples (see Fig. 2). The absence of interfering compounds is indicated by the distribution of 5-HT between the 1-ml fractions, which is the same when acidified urine or 1 ml of 0.3 *M* hydrochloric acid containing 150 ng 5-HT is applied to the XAD-2 column: 50–60% of the 5-HT elutes in the first, 35–50% in the second, 2–4% in the third and 0% in the fourth millilitre (see Fig. 2).

5-HIAA also elutes from XAD-2 (see Fig. 2), but its recovery is low (ca. 30–40% in the first 3 ml), even though the XAD-2 is eluted with a solvent of neutral pH. Using an eluent containing 40% methanol (v/v), 5-HIAA is also quantitatively recovered in 3 ml. However, this extraction procedure was not generally applicable because in some urine samples the determination of 5-HT was hampered by the presence of interfering compounds, whereas in these samples 5-HT could be determined successfully by elution of the XAD-2 column with 20% methanol.

For these reasons the following conditions were chosen for the determination of 5-HT and 5-HIAA: 5-HT was extracted on XAD-2 by elution with 3 ml of water–methanol (8:2, v/v); 50 µl of the eluate were separated, using a mobile phase buffered at pH 4.2 with 0.05 *M* oxalate. A typical chromatogram is shown in Fig. 3A. Oxalate was used instead of phosphate [24] because it has a pK_a of 4.14 [27] and thus it buffers very well at pH 4.2. With the same chromatographic system, 5-HIAA could be determined directly after 1:1 dilution with 0.6 *M* hydro-

chloric acid and filtration (see Fig. 3B). Owing to the presence of indoleacetic acid (IAA), which eluted after ca. 30 min (see Fig. 3B), the analysis was lengthy. However, between 5-HIAA and IAA only some minor peaks were detected. Consequently, the second sample could be injected after elution of 5-HIAA, the third before the elution of the first IAA peak, etc. In this way the effective time per sample was reduced to 15 min. After XAD-2 extraction, IAA was retained on the column (see Fig. 3A) and consequently the analysis time was ca. 10 min.

By applying the appropriate reconditioning procedure the XAD-2 column could be used many times: after ca. 50 extractions 5-HT was still quantitatively recovered in the first 3 ml, although a higher percentage (5–7%) was found in the third millilitre. After another 50 extractions the fourth millilitre of the eluate also contained 5-HT, and the XAD-2 column had to be replaced by a new one.

There is another interesting possibility that can be considered when, instead of the accurate determination of 5-HT and 5-HIAA, a "fingerprint" of the urine concentrations suffices: the untreated urine can be diluted with the eluate of the XAD-2 extraction and an aliquot of this mixture can be analysed by HPLC. Because the concentration of 5-HIAA is much higher than the minimum detectable concentration, only a relatively small volume of untreated urine is required, e.g. one part of urine and ten parts of the XAD-2 eluate. In this way, the analysis time per sample can be reduced by a factor of 2, while generally a good indication of the 5-HT concentration is obtained.

Quantitative aspects

Applying the approach described in the previous section, the linearity of the method was investigated by standard additions to urine at five concentration levels of 5-HT and 5-HIAA up to about five times the normal concentrations. Both for peak height and peak area measurements the standard addition curves were linear, with correlation coefficients of at least 0.9999 for 5-HT and 0.9995 for 5-HIAA. The linearity of the 5-HT curves indicates that the capacity of the XAD-2 column was not exceeded. The recovery of 5-HT after XAD-2 extraction, as calculated from the slopes of the standard addition curve and the calibration curve (peak area measurements) and taking into account the dilution during sample pretreatment, amounted to 96%. The recovery of 5-HIAA, as calculated from the peak areas before and after filtration, was 100%.

The peak shapes of 5-HT and 5-HIAA and, consequently, the peak heights depend on the pH and the methanol content of the solutions injected. This means that quantification by means of peak-height comparison leads to erroneous results, unless prior to HPLC the pH and the methanol content of the standards and processed urine samples are adjusted to the same value.

Relative standard deviations (R.S.D.) for four subsequent 50-μl injections at normal concentration levels (86 ng of 5-HT and 3.4 μg of 5-HIAA/ml urine) were 2.7% for 5-HT and 1.2% for 5-HIAA (peak-area measurements). The R.S.D. for four 5-HT extractions from the same urine sample (duplo injection of each XAD-2 eluate) was 1.2%.

Based on a signal-to-noise ratio of 10, the minimum detectable concentrations in urine were the same for 5-HT and for 5-HIAA, i.e. 20 ng/ml.

Determinations of 5-HT and 5-HIAA in urine

In urine samples from healthy subjects, 5-HT concentrations ranged from 80 to 240 ng/ml and 5-HIAA concentrations from 2 to 8 μg/ml. These concentrations are in good agreement with those reported by other authors [4,6,14,16]. For 5-HIAA the concentrations are much higher than those minimally detectable, while 5-HT can also be determined with good precision even at relatively low concentrations (see Fig. 3).

The concentrations were also determined in urine samples from patients with carcinoid tumours. In 24-h urines 5-HT concentrations ranged from 54 to 3800 ng/ml and 5-HIAA concentrations from 4.0 to 630 μg/ml. Even at these high levels the capacity of the XAD-2 column was not exceeded. This could be concluded from the distribution of 5-HT between the 1-ml fractions of the eluate of the XAD-2 extraction and from reanalysis of a urine sample after a 100-fold dilution. The fourth millilitre of the XAD-2 eluate was collected separately. In none of these fractions was a peak detected with the retention behaviour of 5-HT, indicating that no other compound interfered with its determination.

Stability

The stability of 5-HT and 5-HIAA in concentrated and diluted standards and in urine samples was investigated over 27 days under various storage conditions with respect to the pH (0.7 and 2.0 for standards and 0.8, 2.0 and 6.5 for urine samples), the temperature (−20 and 4°C) and the container material (polypropylene and glass tubes). In order to prevent photodegradation [14], all solutions are stored in containers wrapped in tinfoil.

Generally, only small differences (less than 5%) in stability were found between glass and polypropylene tubes. In the concentrated standard solution 5-HT and 5-HIAA were most stable when stored at pH 0.7 and 4°C, with percentages remaining after 27 days of 98 and 94%, respectively. In diluted standards 5-HT and 5-HIAA were less stable, but stored at pH 0.7 in polypropylene tubes and at 4°C no noticeable degradation of the compounds took place within one week. Apparently, in urine other factors influence the stability of 5-HT and 5-HIAA. At pH 0.8 only ca. 70% of the compounds was left after 27 days. The best results were obtained at physiological pH values: after 27 days 88% 5-HT and 99% 5-HIAA were unchanged when urine was stored at pH 6.5 and −20°C. Since the stability of 5-HT and 5-HIAA was determined in only one urine sample from a healthy person, it cannot be excluded that the stability characteristics are different for other urine samples, e.g. from patients with carcinoid tumours.

CONCLUSIONS

5-HT and 5-HIAA can be determined directly in urine by reversed-phase ion-pair partition chromatography with fluorimetric detection. For 5-HIAA a mobile phase with a pH of 1.95 or 4.2 is used whereas for 5-HT a mobile phase with a pH of 7.2 was selected. Fluorimetric detection proved to be more selective than amperometric detection and was used throughout this study.

Alternatively, both compounds can be determined using the same mobile phase

(pH 4.2), after liquid–solid extraction of 5-HT on a small XAD-2 column. In this clean-up step 5-HT was eluted selectively from the XAD-2 column with water–methanol (8:2, v/v) with a recovery of $96 \pm 1.2\%$ ($n=4$). Standard addition curves were linear up to at least five times the normal urine concentrations. Based on a signal-to-noise ratio of 10, the minimum detectable concentrations of 5-HT and 5-HIAA were both 20 ng/ml in urine. The method is suited to the assay of 5-HT and 5-HIAA in urine at normal and increased concentration levels.

Concentrated and diluted standards of 5-HT and 5-HIAA were stable for one month and one week, respectively, if stored in 0.3 *M* hydrochloric acid (pH 0.7) at 4°C. Preliminary results seem to indicate that urine samples are most stable if stored frozen (−20°C) at their physiological pH values, but the assay should take place within one month after sampling.

ACKNOWLEDGEMENTS

Professor Dr. A.T. van Oosterom and the nursery staff of the Department of Clinical Oncology, Medical Centre, University Hospital, Leiden, are thanked for supplying us with the urine samples from the carcinoid syndrome patients.

REFERENCES

1 W.C. Bowman and M.J. Rand, Textbook of Pharmacology, Blackwell, Oxford, 2nd ed., 1980, Ch. 12, p. 20.
2 A.P. Graffeo and B.L. Karger, Clin. Chem., 22 (1976) 184.
3 J.P. Garnier, B. Bousquet and C. Dreux, J. Chromatogr., 204 (1981) 225.
4 K.-G. Wahlund and B. Edlén, Clin. Chim. Acta. 110 (1981) 71.
5 K.-G. Wahlund and B. Edlén, J. Chromatogr., 204 (1981) 269.
6 M. Goto, E. Sakurai and D. Ishii, J. Liq. Chromatogr., 6 (1983) 1907.
7 V. Skrinska and S. Hahn, J. Chromatogr., 311 (1984) 380.
8 A. Iwatani and H. Nakamura, J. Chromatogr., 309 (1984) 145.
9 J.P.M. Wielders and C.J.K. Mink, J. Chromatogr., 310 (1984) 379.
10 R.F. Seegal, K.O. Brosch and B. Bush, J. Chromatogr., 377 (1986) 131.
11 O. Beck, G. Palmskog and E. Hultman, Clin. Chim. Acta, 79 (1977) 149.
12 R.P. Tracy, L.E. Wold, J.D. Jones and M.F. Burritt, Clin. Chem., 27 (1981) 160.
13 T.G. Rosano, J.M. Meola and T.A. Swift, Clin. Chem., 28 (1982) 207.
14 N. Fornstedt, Anal. Chem., 50 (1978) 1342.
15 N. Fornstedt, J. Chromatogr., 181 (1980) 456.
16 D.D. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
17 B.H.C. Westerink, F.J. Bosker and J.F. O'Hanlon, Clin. Chem., 28 (1982) 1745.
18 D. Tonelli, E. Gattavecchia and M. Gandolfi, J. Chromatogr., 231 (1982) 283.
19 P.P. Chou and P.K. Jaynes, J. Chromatogr., 341 (1985) 167.
20 J. Segura, F. Artigas, E. Martinez and E. Gelpi, Biomed. Mass Spectrom., 3 (1976) 91.
21 E. Kwarts, J. Kwarts and H. Rutgers, Ann. Clin. Biochem., 21 (1984) 425.
22 J. Jouve, J. Martineau, N. Mariotte, C. Barthelemy, J.P. Muh and G. Lelord, J. Chromatogr., 378 (1986) 437.
23 T.P. Davis, G.W. Gehrke, C.W. Gehrke Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, J. Chromatogr., 162 (1979) 293.
24 J. de Jong, U.R. Tjaden, W. van'tHof and C.F.M. van Valkenburg, J. Chromatogr., 282 (1983) 443.
25 I. Morita, T. Masujima, H. Yoshida and H. Imai, Anal. Biochem., 118 (1981) 142.

26 C. van Valkenburg, U. Tjaden, J. van der Krogt and B. van der Leden, J. Neurochem., 39 (1982) 990.
27 W.P. Jencks and J. Regenstein, in H.A. Sober (Editor), Handbook of Biochemistry, CRC, Cleveland, OH, 1st ed., 1968, p. J154.