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Rapid measurement of the monoamine content in small volumes of rat plasma

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Abstract

A method for the simultaneous measurement of serotonin, catecholamines, and their metabolites, 5-hydroxyindoleacetic acid, homovanillic acid, and 3, 4-dihydroxyphenylacetic acid, by ultrafiltration and microbore high-performance liquid chromatography with dual electrochemical detection in small plasma volumes was established. Unlike the traditional assays which require at least 1–2 ml of plasma for each measurement, the present method uses only a 20-μl sample volume. Since blood loss is minimal, repeated blood sampling from a single animal as a rat becomes practicable. The present microassay provides low detection limits (signal-to-noise ratio = 3) for all analytes (0.2–0.5 pg per 5-μl injection or 50–120 pg/ml plasma). Isocratic separation of these analytes on a microbore column is achieved within 15 min. This rapid and sensitive method can be used as a routine research tool in various physiological or pharmacokinetic studies especially in small animals.

1. Introduction

Measurement of plasma serotonin, catecholamines, and their metabolites is commonly used in the evaluation of the drug-treatment of psychiatric diseases [1,2] and in the study of the role of the autonomic nervous system in various physiological or pathophysiological conditions in animal models [3,4]. Many analytical procedures have been devised for these purposes [5–8]. High-performance liquid chromatography with electrochemical detection (HPLC-ED) is one of most popular methods because of its reasonable versatility, sensitivity, and specificity [9]. In general, traditional HPLC-ED methods used for the determination of these compounds in plasma samples need extensive and time consuming clean-up procedures [10–13]. This is due to the large number of interfering substances in plasma appearing in traditional chromatography. Until recently, plasma sample clean-up procedures have been the rate-limiting step in a routine laboratory. Most published HPLC assays for catecholamines use plasma sample volumes of 1–2 ml (or 3–5 ml blood samples) [14–17]. The repeated withdrawal of such large sample volumes raises problems, especially in experiments with small animals like rats.

Ultrafiltration techniques have been used for plasma processing in drug monitoring assays

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[18,19]. In our recent study, ultrafiltration has been successfully applied in assaying human plasma serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) [20]. Unfortunately, the large elution front gives rise to small peaks of norepinephrine (NE), epinephrine (E) and/or DOPAC in anodic chromatograms. The solvent front peaks can be readily eliminated by applying a series dual electrochemical detector giving well isolated peaks of NE, E and DOPAC in the cathodic chromatograms [21]. The objective of this work was to apply ultrafiltration to very small plasma samples (<100 μl) of rats prior to HPLC with dual electrochemical detection for the simultaneous measurement of NE, E, DOPAC, dopamine (DA), 5-HIAA, HVA, and 5-HT.

2. Experimental

2.1. Apparatus and chromatographic conditions

The biogenic amines were analyzed by HPLC-ED as described previously [20,21]. Briefly, the HPLC system consisted of a Beckman pump (Beckman Instruments, Taiwan Branch, Taiwan), a CMA-200 microautosampler (CMA/Microdialysis, Stockholm, Sweden), two BAS-4C amperometric detectors (Bioanalytical Systems, West Lafayette, IN, USA), a microbore reversed-phase column (Inertsil-2, 5-μm ODS, 150 × 1.0 mm I.D., G.L. Sciences, Tokyo, Japan), and the Beckman System Gold Data Analysis Software (Beckman Instruments). The anodic and cathodic glassy carbon working electrodes were set at +0.75 V and +0.05 V with respect to a silver/silver chloride reference electrode, respectively [21]. The mobile phase was slightly modified compared with that used in our previous study [21] and consisted of 80 ml acetonitrile, 5 ml tetrahydrofuran (THF), 0.48 g sodium 1-octane sulfonate (SOS) (2.6 m*M*), 2.00 g monosodium dihydrogen orthophosphate (14.7 m*M*), 8.82 g sodium citrate (30 m*M*), 10 mg ethylenediaminetetraacetic acid EDTA (0.027 m*M*), and 1 ml diethylamine in double distilled water. The pH of the solution was adjusted to 3.5 and the flow-rate was 60 μl/min maintaining the column pressure at *ca.* 10.3 MPa. NE, E, DA, DOPAC, 5-HIAA, HVA, 5-HT, 3-methoxytyramine (3-MT, internal standard [20]), EDTA, diethylamine, SOS, monosodium dihydrogen orthophosphate, picrotoxin, and sodium citrate were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and THF were purchased from Merck (Merck-Schuchardt, Darmstadt, Germany). All reagents were analytical grade unless otherwise stated.

2.2. Sample preparation and assay

Standard stock solutions of NE, E, DOPAC, DA, HVA, 5-HT, 5-HIAA, and 3-MT were prepared at a concentration of 2 ng/ml in 0.1 *M* perchloric acid and stored at −70°C in the dark. For the daily preparation of a standard mixture a portion of these stock solutions was thawed at 4°C and diluted to the appropriate concentration with Ringer solution containing 10^{-7} *M* ascorbic acid in 0.1 *M* HCl. Seven WKY rats (300–350 g) were anaesthetized with urethane (1.2 g/kg), intraperitoneally. A cannula (PE 90, polyethylene tubing) was placed in the carotid artery for the collection of blood samples. Three sequential blood samples (0.2 ml each) were collected prior to administration of picrotoxin (PX, 10 mg/kg, i.v.) in saline or saline alone (10 μl/min throughout the experiment) were given. Additional blood samples were taken at 3, 5, 8, 13, 23, and 30 min after PX injection or at 5-min intervals prior to saline injection in each rat. Blood samples were collected into pre-chilled micro-polypropylene vials and centrifuged (10 min, 700 *g* at 4°C) immediately to separate the plasma. Frozen plasma samples were kept at −70°C prior to assay. After thawing at 4°C, 100 μl plasma sample was transferred into a Millipore Ultrafree-MC unit and centrifuged at 15 000 *g* for 15 min [21]. A 40-μl aliquot of the plasma ultrafiltrate and 10 μl Ringer solution containing 1.4 ng 3-MT (as internal standard) and 10^{-7} *M* ascorbic acid (as an anti-oxidant) were mixed. In addition, ultrafiltration procedures were carried out on 20–200 μl pooled plasma to investigate the precision and accuracy of this microassay. Plasma ultrafiltrate concentrations of NE, E,

DOPAC, DA, HVA, 5-HT, and 5-HIAA were calculated by determining the peak-area ratio relative to the internal standard 3-MT, and adjusted to plasma concentrations according to the recovered volume for each plasma sample. The identity of the peaks in the chromatograms was confirmed by their retention times, addition of standards, redox ratios, and a superimpose–alignment technique provided by the System Gold Data Analysis Software from Beckman.

3. Results and discussion

Fig. 1A and B show typical anodic and cathodic chromatograms of a standard mixture containing NE, E, DOPAC, DA, HVA, 5-HIAA, 3-MT (internal standard), and 5-HT. Separation was completed within 15 min. The known amounts (5 pg–20 ng per 5 μl injection) of all analytes in the standard mixtures were linearly related to the cathodic (for NE, E, DOPAC, and DA) or anodic (for 5-HIAA, HVA, 3-MT and 5-HT) chromatographic areas obtained from the standard mixture ($R^2 \geq 0.999$). This is in accordance with our previous study where the concentrations of NE, E, DOPAC, and DA were determined from the cathodic chromatogram, while 5-HIAA, HVA, 3-MT, and 5-HT were determined from the anodic chromatogram using the HPLC-ED system [21]. Amounts of these analytes in the range of 1–5 pg per 5 μl injection were also linearly related to their chromatographic areas ($R^2 \geq 0.980$).

The precision of the assays was tested using a pooled rat plasma (containing 1–8 ng/ml of each analyte) and a standard mixture (containing *ca.* 100 pg of each analyte) in 0.1 *M* HCl and Ringer solution containing 10^{-7} *M* ascorbic acid. The intra- ($n = 8$) and inter-assay ($n = 6$) variabilities were assessed and expressed as coefficients of variation (C.V., %) and are shown in Table 1. The intra-assay variability was assessed at intervals of 1 h using a standard mixture. 5-HIAA gave a relatively high C.V. value (4.88 %) while the others were satisfactory (≤3.87%). In plasma ultrafiltrates the C.V.s were excellent for 5-HIAA and HVA and acceptable for the other compounds (≤7.12%). In general, rat plasma DA levels were very low or below the detection limit of the present method. The variability in the quantitation of the analytes in plasma samples usually depends on their concentration and on the occurrence of interfering peaks in the chromatograms. Most interfering substances can

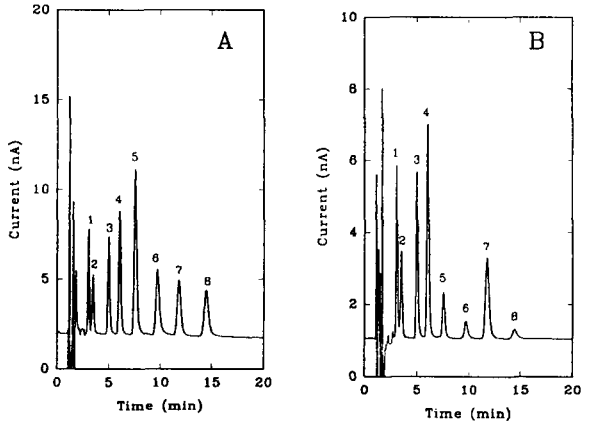

Fig. 1. Typical chromatograms of a standard mixture containing (1) NE, 166 pg/inj; (2) E, 121 pg/inj; (3) DOPAC, 198 pg/inj; (4) DA, 224 pg/inj; (5) 5-HIAA, 394 pg/inj; (6) HVA, 218 pg/inj; (7) 3-MT (internal standard), 193 pg/inj; (8) 5-HT, 179 pg/inj. (A) anodic current, (B) cathodic current, applied potentials (*vs.* Ag/AgCl): anode (+) 0.75 V, cathode (+) 0.05 V.

Table 1
Intra-assay ($n = 8$, at 1-h intervals) and inter-assay ($n = 6$, on six consecutive working days) stability for a standard mixture and a pooled rat plasma ultra filtrate (HPLC-ED).

Compound	Coefficient of variation (%)		
	Intra-assay		Inter-assay
	Standard mixture	Plasma	Standard mixture
NE	3.40	7.12	2.84
E	1.74	7.09	1.46
DOPAC	1.87	4.62	4.49
DA	2.45	N.D.[a]	2.68
5-HIAA	4.88	1.68	4.96
HVA	3.87	1.74	1.07
5-HT	2.16	5.22	6.40

[a]N.D. = Not detectable.
Standard mixtures consist of *ca.* 100 pg catecholamines, serotonin, and their metabolites.

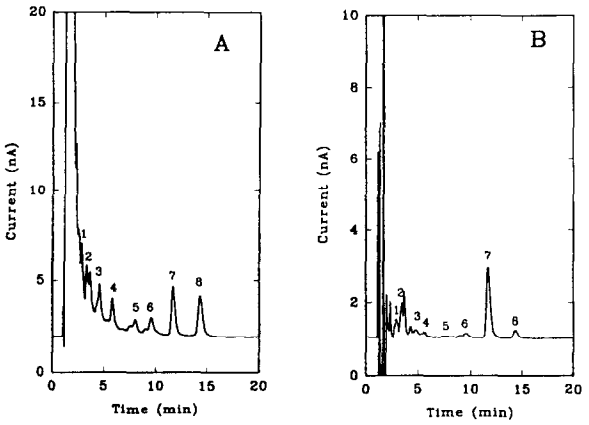

Fig. 2. Typical chromatograms of an ultrafiltrate of a rat plasma sample at basal level containing (1) NE; (2) E; (3) DOPAC; (4) DA; (5) 5-HIAA; (6) HVA; (7) 3-MT (as an internal standard); (8) 5-HT. (A) anodic current, (B) cathodic current.

be removed by the alumina extraction method. Unfortunately, it is difficult to remove these interfering substances by a simple ultrafiltration procedure. However, quantitation of the analyte peaks has been improved by the microbore column separation and the dual electrochemical detection used in the present method.

Fig. 2A and B show typical chromatograms of a rat plasma ultrafiltrate. Peaks of NE, E, DOPAC, DA, HVA, 5-HIAA, 3-MT, and 5-HT in Figs. 2A and B were identical to those in Figs. 1A and B. However, there were several interfering peaks around NE and E in the anodic chromatogram as shown in Fig. 2A. These interfering peaks were dramatically reduced in the cathodic chromatogram as shown in Fig. 2B. Therefore, a more reliable assignment of the NE and E peaks could be achieved by measuring those from the cathodic chromatograms. The inter-assay variabilities assessed with a standard mixture on six consecutive working days were less than 6.40% for all analytes. The detection limits (signal-to-noise ratio = 3) of all analytes per 5-μl injection were between 0.2 and 0.5 pg (or 50–120 pg/ml plasma content).

In our previous study, good volume recoveries ($n = 6$, $98 \pm 2\%$) of these analytes after ultrafiltration of standard mixtures were found. There is no need to correct the recoveries of the ultrafiltrate samples. Under the same conditions, more than 54% ($n = 9$, $55 \pm 2\%$) of the rat plasma sample volumes was recovered. The plasma concentrations can be obtained by correction according to HPLC-ED assay, its diluting factor, and the measured volume recovered in the ultrafiltration procedure. The recovery of the monoamine content and the intra-assay coefficient of variation of the ultrafiltration procedures ($n = 3$) performed in Millipore Ultrafree-MC units with a pooled plasma (with plasma content 1–8 ng/ml) are shown in Table 2. Variation of the ultrafiltrate volume in triplicate plasma samples (20–200 μl) was less than 4%. Plasma

Table 2
Recovery, monoamine content (ng/ml), and intra-assay ($n = 3$) coefficient of variation (C.V., %) of ultrafiltration procedures in Millipore Ultrafree-MC units for a pooled plasma in different plasma volumes.

Plasma volume (μl)	20	30	40	50	60	80	100	120	150	200	C.V.[a] (%)	C.V.[b] (%)
Filtrate-volume (μl)	11.5	16.5	25.0	31.0	34.0	44.5	55.5	60.0	70.0	76.0	<3	<4
Recovery (%)	58.7	55.0	62.5	62.0	56.7	55.6	55.5	50.0	46.7	38.0	5.4	13.7
NE	4.01	4.04	4.09	4.13	4.16	4.09	4.22	4.13	4.20	4.09	1.71	1.58
E	4.44	4.46	4.50	4.66	4.70	4.75	4.87	4.77	4.85	4.68	3.54	3.33
DOPAC	2.80	2.86	2.85	2.70	2.69	1.17	2.61	2.60	2.58	2.50	3.53	4.44
5-HIAA	1.07	1.04	1.05	1.03	1.02	1.02	0.98	1.00	1.01	0.93	2.71	3.78
HVA	1.60	1.71	1.77	1.90	1.86	1.77	1.80	1.74	1.70	1.69	5.57	5.00
5-HT	7.74	7.73	7.75	7.81	7.77	7.77	7.73	7.70	8.04	8.04	0.36	1.58

[a] C.V. of plasma volume between 20–100 μl.
[b] C.V. of plasma volume between 20–200 μl.

volumes larger than 200 μl showed insufficient filtration in our preliminary study. Furthermore, in order to preserve the HPLC injection precision, to overload a 5 μl sample loop (>10 μl), to overcome the hold-up volumes of the Millipore Ultrafree-MC · units (*ca.* < 5 μl) and of our CMA-200 autosampler (*ca.* < 1 μl), the required volume of a plasma sample is estimated to be at least 20 μl. Therefore, 20–200 μl plasma volumes were used to investigate the precision and accuracy of the ultrafiltration procedures. The recoveries of ultrafiltrates (55.5–56.7%) were consistent with plasma volumes in the range of 60–100 μl. Satisfactory results were also obtained between 20–50 μl (55–62%). Increasing the plasma sample volume above 200 μl reduced the volume recovered after ultrafiltration to less than 40%. This phenomenon might be due to clogging of the membrane by high-molecular-mass proteins or substances resulting in insufficient ultrafiltration. Indeed, in some cases plasma suspensions were observed on top of the membrane after centrifugation when the plasma volumes were greater than 100 μl. However, a 20 μl plasma sample was sufficient to obtain data as precise and accurate (C.V. ≤ 5% for all analytes) as those obtained with a 100 μl volume in the ultrafiltration assay of the present study. These data also indicate satisfactory reproducibility and analytical precision of pooled plasma sample volumes in the range 20–100 μl (C.V. ≤ 5.4%). The ability to measure plasma contents in a small volume has a great analytical potential in studies where repeated blood sampling is necessary or in pharmacokinetic studies in small animals such as rats.

Even if the retention characteristics and the current–potential behavior of a sample chromatographic peak compare well with those of a standard compound, unreliable measurements could occur with a single electrode detector. In the present study, each peak was also verified by spiking the standard mixture, and observing if the addition increased its peak height but did not change its shape and retention time. In addition, information on the anodic and cathodic chromatographic peaks can be obtained by the sequential performance of a dual electrochemical detector. The redox ratios (reductive peak current/oxidative peak current) of the analytes in the plasma ultrafiltrates were recorded and compared with those of the authentic standards. In general, peak identity was confirmed when the variation of the redox ratio was below 10%. Redox ratios provide additional information on peak purity.

Unfortunately, the redox ratios of the analytes in the plasma ultrafiltrates are not exactly in agreement with those obtained with the authentic standards. For instance, the redox ratios of NE and E found in this study for the series dual electrode were 0.85 and 0.80, respectively. From the chromatographic peaks of plasma ultrafiltrates these values were found to be 0.25 for NE and 0.44 for E. Obviously, we were unable to confirm the purity of the NE and E peaks which might be co-eluted with some interfering peaks in the anodic chromatograms (Fig. 2A). The interfering peaks could increase the peak area in the anodic chromatogram and give rise to decreasing redox ratios for the NE and E peaks, if the interfering peaks do not represent electrochemically reversible species. Fortunately, these plasma contaminants were usually not detected in the cathodic chromatograms in our routine assay. Therefore, clean chromatographic NE and E peaks were obtained in the cathodic chromatogram of plasma ultrafiltrates, together with clean DOPAC and DA peaks. Hence, NE, E, DOPAC, and DA in plasma ultrafiltrates could be accurately measured from the clean cathodic chromatograms. Redox ratios of authentic HVA and 5-HT utilized in this study for the series dual electrode were 0.22 and 0.11, respectively. From the chromatographic peaks of plasma ultrafiltrates these values were found to be 0.21 for HVA and 0.10 for 5-HT. We were able to confirm the purity of plasma HVA and 5-HT.

A superimpose–alignment technique may be useful when the elution time of the chromatographic peaks are slightly different between runs or when they co-elute with other unknown interferences. The chromatograms are adjusted to align according to the differences between some selected peaks and the internal standard (3-MT) by the Beckman Gold System. Some-

times superimposition of the chromatograms is valuable in confirming and aligning peaks from different runs. This technique can also be used for comparing chromatograms obtained from different physiological stages in the same experimental subject.

The effects of injection of PX on plasma biogenic amines and their metabolites were investigated by the present method. The first blood sample was discarded because of possible operational damage or contamination with tissue debris during preparation. No significant difference in plasma content at the different stages was found in control rats (Table 3). These data indicated that the plasma levels of serotonin, catecholamines and their metabolites generally did not change significantly. Plasma 5-HIAA levels in the last two stages were significantly higher ($p < 0.05$, analyzed by Fisher PLSD test) than that in the first stage during 2–3 ml blood drawing with an additional perfusion of 1 ml saline in our experimental protocol. The mechanism which caused this variation is unknown. However, the minimal effect on plasma content strongly encourages further pharmacological or physiological applications of the present method in repetitive blood drawing experiments in small animals. Compared to the basal plasma levels, PX injection caused a significant elevation ($p < 0.05$) of plasma serotonin, cathecholamines, and their metabolites at different stages (Table 3). These results are in agreement with our previous experiments [22–24].

The plasma pretreatment of the present method took less than 30 min for 16 samples, whereas others, *e.g.* traditional alumina or liquid–liquid extraction procedures [25,26], would take a few hours for 6 samples. Although clean chromatographic peaks are obtained by alumina extraction, this goes at the expense of time-consuming pretreatment procedures. The variation in the recovery is still a major problem for routine alumina extraction methods in inter-laboratory comparison [27]. In addition, the present assay is suited for the simultaneous measurement of serotonin, catecholamines, and their metabolites, whereas with the alumina extraction method only measurements of catecholamines are

Table 3
Plasma serotonin, catecholamines, and their metabolites (ng/ml) levels in rats with ($n = 4$) or without ($n = 3$) PX injection measured by the HPLC-ED system

Stage[a]	NE		E		DOPAC		5-HIAA		HVA		5-HT	
	Control	PX	Control	PX	Control	PX	Control	PX	Control	PX	Control	PX
1	0.57 ± 0.20	0.71 ± 0.11	2.87 ± 0.93	3.14 ± 0.49	0.67 ± 0.21	0.76 ± 0.06	16.78 ± 7.87	16.22 ± 7.21	3.55 ± 0.44	4.01 ± 0.38	9.65 ± 4.72	10.25 ± 3.93
2	0.55 ± 0.22	0.93 ± 0.15	3.19 ± 1.14	3.14 ± 0.60	0.78 ± 0.20	0.70 ± 0.13	19.03 ± 9.22	11.83 ± 2.84	3.76 ± 0.47	4.08 ± 0.62	8.53 ± 4.86	7.55 ± 1.66
3	0.64 ± 0.22	0.99 ± 0.16	3.51 ± 1.13	3.05 ± 0.44	0.81 ± 0.20	0.71 ± 0.27	22.22 ± 11.44	11.36 ± 1.90	4.25 ± 0.57	4.75 ± 0.63	10.12 ± 8.90	8.43 ± 2.81
4	0.78 ± 0.35	0.98 ± 0.15	3.70 ± 1.17	2.79 ± 0.17	0.89 ± 0.25	1.00 ± 0.05	24.69 ± 13.61	11.59 ± 2.50	4.55 ± 0.71	5.41 ± 0.88	8.61 ± 4.83	18.68 ± 5.19
5	0.77 ± 0.29	1.80 ± 0.23	3.95 ± 1.07	3.13 ± 0.41	0.90 ± 01.5	1.40 ± 0.14	27.54 ± 15.29	18.43 ± 6.98	5.06 ± 0.78	5.24 ± 0.83	7.25 ± 2.35	14.45 ± 4.31
6	0.68 ± 0.22	2.88 ± 0.67	4.10 ± 0.95	5.60 ± 2.88	1.00 ± 0.27	1.78 ± 0.41	28.04 ± 15.50	19.18 ± 5.37	5.29 ± 0.67	5.65 ± 0.48	5.90 ± 1.20	25.37 ± 6.94
7	0.81 ± 0.36	3.19 ± 0.50	3.84 ± 1.24	5.59 ± 2.13	0.91 ± 0.34	1.60 ± 0.16	28.89 ± 15.72	13.84 ± 3.15	5.55 ± 0.78	5.87 ± 1.72	22.72 ± 9.66	11.57 ± 2.07
8	0.60 ± 0.18	6.41 ± 1.60	2.45 ± 0.74	13.55 ± 4.54	0.98 ± 0.39	3.42 ± 0.67	39.83 ± 20.76	23.42 ± 10.19	6.95 ± 0.99	8.41 ± 2.42	8.56 ± 4.73	21.12 ± 9.17
9	0.74 ± 0.15	7.47 ± 3.87	2.25 ± 0.55	15.08 ± 8.06	1.16 ± 0.58	5.21 ± 2.96	39.64 ± 20.90	27.81 ± 9.42	6.87 ± 1.02	10.53 ± 4.10	8.57 ± 4.69	30.89 ± 2.67

[a] Three blood samples were taken (5-min intervals) prior to administration of PX or saline. Additional blood samples were taken at 3, 5, 8, 13, 23, and 30 min after PX administration or at 5-min intervals for the control group.

feasible. The present method uses plasma volumes as low as 20 μl, which is impossible with the alumina extraction method. In this study, automated HPLC analysis can be used when many plasma samples and/or very small volume plasma samples have to be analyzed in a short period of time. Another advantage of this microassay is the low detection limit for the analytes (0.2–0.5 pg per 5-μl injection, or 50–120 pg/ml plasma volume). Furthermore, complete separation of the analytes within 15 min was achieved. Hence over 90 analyses can be done in one working day by our automated HPLC system.

4. Conclusions

The microassay presented in this paper is rapid as compared to some complicated traditional methods, such as alumina or liquid–liquid extraction assays used for the measurement of plasma serotonin, catecholamines, and their metabolites in small volumes. The identification of the peaks and the liability of the measurements can be improved by dual electrochemical detection. In addition, very sensitive detection was achieved by HPLC-ED with microbore columns. We conclude that this microassay is a suitable tool for pharmacokinetic or physiological experiments in small animals.

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