Automated analysis of homovanillic acid and 5-hydroxyindole-3-acetic acid in biological fluids by coupled-column liquid chromatography and dual coulometric–amperometric detection

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Abstract: Homovanillic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were determined by direct injection of plasma, urine and cerebrospinal fluid (CSF) into a liquid chromatographic system comprising three columns, one packed with an anion-exchange gel and two with reversed-phase material. The acidic compounds were isolated by anion-exchange on the first column, enriched on the second and separated by reversed-phase chromatography on the last column. 5-HIAA and HVA were detected by coulometry and amperometry on two working electrodes coupled in series, giving limits of determination in plasma of 4 and 5 nmol l^{-1} respectively. Endogenous plasma, urine and CSF-levels were determined with a precision (RSD) of 2–4%. 3-Methoxy-4-hydroxyphenylpropionic acid was identified in normal plasma and urine.

Keywords: *Anion exchange precolumn; trace enrichment; reversed phase chromatography; electrochemical response ratios; biological samples; direct injection.*

Introduction

There is considerable interest in quantitative methods for catecholamines, 5-hydroxytryptamine and their metabolites in brain tissue and biological fluids. Quantitative determinations are required in many different studies, the most important being studies of neurochemical abnormalities, the effects of drug treatments and the diagnosis of cancerous tumors. A large number of methods have been described and several review articles and books are available on the subject [1–4]. Sensitive methods for the determination of the acidic metabolites have been realized by gas chromatography–mass spectrometry and radioenzymatic techniques. These methods are laborious. On the other hand liquid chromatography with amperometric or fluorimetric detection may be used to provide fast and sensitive methods. Urine and cerebrospinal fluid samples are often injected directly onto liquid chromatography columns for the determination of 5-hydroxyindole-3-acetic acid [5, 6], homovanillic acid [7] or both [8, 9], whilst plasma proteins are separated by precipitation prior to injection [10–12]. Assays with direct injection of biological fluids or crude extracts may be sensitive to variations of the matrix composition, therefore it is desirable to combine the chromatographic separation with

some form of selective isolation procedure. Liquid–liquid extractions have been used [13, 14], but the recoveries are usually low and variable. Chromatographic isolation on anion exchangers also has been used [15, 16].

Methods for the isolation and detection of 5-hydroxyindole-3-acetic acid and homovanillic acid, compatible with column switching and with efficient chromatographic systems, have been studied in the present work. Suitable conditions for direct injection of plasma, urine and CSF on anion-exchange gels have been developed. The selectivity of the system has been evaluated by comparison of the response ratio at two different working potentials.

Experimental

Chemicals and reagents

5-Hydroxyindole-3-acetic acid (5-HIAA), 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA), 3-methoxy-4-hydroxyphenylpropionic acid (MOPPA) and glutathione were obtained from Sigma (St Louis, MO, USA). 5-Hydroxyindole-3-propionic acid (5-HIPA) was obtained from Labkemi (Gothenburg, Sweden), N,N-dimethyl-N-octylamine (DMOA) was obtained from ICN Pharmaceuticals Inc (Plainview, NY, USA). Methanol and buffers were all of analytical grade quality. Water was deionized in an Elgstat Spectrum water purification system (Lane End, Buckinghamshire, UK).

Apparatus

The liquid chromatograph consisted of an automatic injector, model MSI 660 (Kontron AG, Zürich, Switzerland) having three six-port pneumatic valves with solenoid interfaces (Autochrom, Milford, MA, USA). The switching events were controlled by an integrator, model PU 4810, with an external controls option (Pye Unicam Ltd, Cambridge, UK). The first column (18 × 3.8 mm i.d.) was packed with aminoethyl Bio-Gel P2 (Biorad Laboratories, Richmond, CA, USA). The second column (20 × 3.8 mm i.d.) was packed with 5-μm Nucleosil ODS (Machery-Nagel, Düren, FRG) and the last column (100 × 4.6 mm i.d.) was packed with 3-μm Spherisorb ODS-2 (LKB, Bromma, Sweden). The last column was connected to a coulometric detector, Coulochem model 5100A with cell model 5011 (Environmental Science Assoc. Inc., Bedford, MA, USA). The mobile phases were pumped by means of two Milton Roy minipumps and a LKB-2150 pump for the precolumns and main column, respectively. A diagram of the system is presented in Fig. 1.

Chromatographic system (see Fig. 1)

C1: Anion-exchange column. A polyacrylamide gel substituted with aminoethyl groups (aminoethyl Bio-Gel P2) was used for the isolation of acidic compounds. The gel was swollen in mobile phase (75 mM acetate buffer) and packed in a precolumn (18 × 3.8 mm i.d. Waters Assoc. Inc., Milford, MA, USA). The internal diameter was enlarged at both ends in order to accept two wide-pore polymeric filters obtained from liquid–solid extraction columns (Bond Elut®, Analytichem). A 0.5 mm hole was made in the original filters supplied with the column. During injection the mobile phase consisted of 75 mM acetate buffer pH 5.5. The flow-rate was set to 0.63 ml min^{-1} by pump 1. A step gradient of phosphate buffer, $\mu = 0.1$ and pH 2.5, was generated by means of loop 2 (2.2 ml) by switching valve 2.
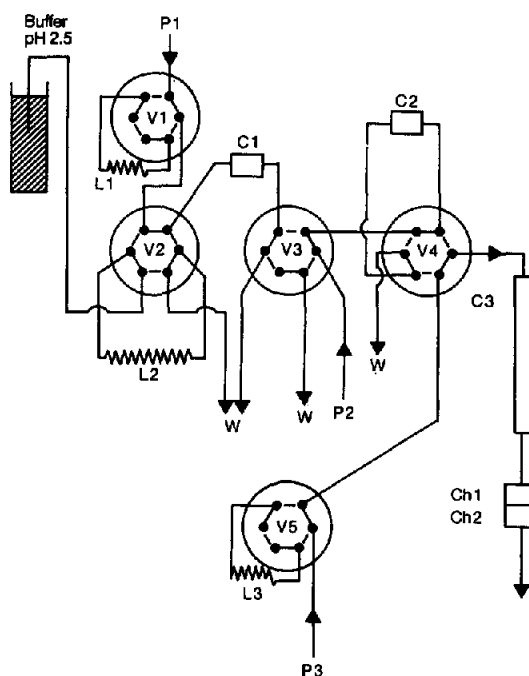
Figure 1
Column-switching system. L 1 = 0.20 ml, L 2 = 2.2 ml and L 3 = 20 µl (for direct injection to the main column). V1 = automatic injector, V2, V3 and V4 = pneumatic valves, V5 = manual valve for direct injections.

C2: Enrichment column. The acids were eluted with an acidic phosphate buffer from the anion-exchange column and were enriched on a short reversed-phase column. A precolumn packed with octadecylsilica (5 µm Nucleosil ODS, 20 × 3.8 mm i.d.) was used for enrichment. The acids were eluted with a mobile phase containing 18% v/v methanol (see main column below). The flow-rate was set to 1 ml min^{-1} by pump 2.

C3: Main column. A reversed-phase column (3-µm Sperisorb ODS-2, 100 × 4.6 mm i.d.) was used for the separation of the different acids. The mobile phase consisted of phosphate buffer (μ = 0.1, pH 2.5)–methanol (82:18, v/v) with 0.5 mM DMOA. The flow-rate was set to 1 ml min^{-1} by pump 3.

Column switching
Plasma, diluted urine or CSF (0.2 ml) was injected directly onto column 1 for 1 min after switching valve 1. The acids were eluted onto the enrichment column by flushing with an acidic buffer for 2.4 min. The acids were then eluted from the enrichment column to the main column by back-flushing, with a buffer containing 18% v/v methanol, for 0.5 min. These switching events are presented in Table 1. Recoveries and band-broadening effects of the column switching system were determined by comparison of peak areas and peak widths after direct injection onto the main column by valve 5.

Table 1
Scheme of column-switching events

Time after injection (min)	Valve operated	Event
0.0	1	Sample is injected onto the anion-exchange gel column with acetate buffer pH 5.5
1.0	2	Acids are eluted from the anion-exchanger with phosphate buffer pH 2.5
1.2	3	Column 1 and 2 are connected in series and the acids are enriched on the reversed-phase column
3.6	3 Reset	Column 1 and 2 are isolated from each other
3.61	4	The sample is back-flushed from the enrichment column onto the main column with a buffer containing 18% v/v methanol
4.11	4 Reset	The elution from the enrichment column is completed and the acids are separated on the main column
6.0	2 Reset	The anion-exchanger is conditioned with acetate buffer pH 5.5
18.0	End	Calculation and report
19.0		The next sample is injected

Coulometric detection (Ch1, Ch2)

The coulometric cell comprises two separate working electrodes coupled in series. Channels one and two were operated at +0.3 V and +0.44 V, versus the reference, respectively.

Channel one was used for coulometric detection of 5-HIAA and channel two for amperometric detection of HVA.

Sample collection and storage

Venous blood samples were collected in heparinized Venoject® tubes (Leuvren, Belgium). The blood was centrifuged and the plasma stored in polypropylene tubes at −70°C. Urine samples were acidified with hydrochloric acid to pH 2.5–3.5 and stored in polypropylene tubes at −70°C.

Standards for calibration

CSF and urine samples were standardized by calibration with 5-HIAA, 5-HIPA and HVA diluted in 75 mM acetate buffer (pH 5.5) with 1 mM glutathione. Plasma standards for calibration were prepared by standard addition.

The response factors for 5-HIAA and HVA in normal blank plasma were determined by standard addition of known amounts (0–1000 nmol l^{-1}) of 5-HIAA and HVA. An aliquot (0.1 ml) of a standard solution containing 5-HIAA, HVA and 5-HIPA was mixed with 0.9 ml of normal blank plasma. The response for the endogenous (blank) level was determined in triplicate and six standard additions were made. The slope of the calibration curve was estimated by least square linear regression analysis of the 'concentrations added' (amount added/final volume) versus peak height ratios.

The unknown endogenous concentration (X) was calculated from the regression equation:

X = Peak height ratio/(slope $\times$ F)
F = Dilution factor (0.9).

The normal plasma was spiked to contain 200–300 nmol l^{-1} of 5-HIAA and HVA. The spiked plasma was used for the calibration of unknown plasma samples and the normal blank plasma for control samples respectively. The standards were stored at $-70°C$. Artificial CSF was prepared as described previously [17].

Sample preparation

The samples were thawed, centrifuged and then diluted with 75 mM acetate buffer pH 5.5, containing internal standard (5-HIPA) and antioxidant (glutathione). Urine samples were diluted 1:50. CSF samples were mixed with an equal volume of buffer, plasma samples (0.45 ml) were mixed with 0.05 ml of acetate buffer. The final concentration of glutathione was 1 mM.

Results and Discussion

Pre-separation and enrichment

When precolumns are used to improve the selectivity of reversed-phase systems, the highest selectivity is obtained when different retention principles pertain to the different columns. Furthermore, the eluent from the first column must be a weak eluent with respect to the following column in order to create enrichment effects. Different anion-exchangers were tested for isolation of HVA, DOPAC and 5-HIAA. The retention volumes on silica based anion-exchangers were unstable. The peaks were asymmetric, and 5-HIAA was strongly adsorbed on the silica matrix during elution with acid. The ion-exchange gel, Aminoethyl Bio-Gel P2, gave symmetrical peaks with complete separation of HVA and of 5-HIAA from neutral matrix components (Fig. 2, Table 2). The non-specific adsorption to the gel matrix was low and the analytes were desorbed with a small volume of an acidic aqueous buffer. 5-HIAA and HVA were well retained by octadecylsilica under acidic conditions and an enrichment factor (k' during injection/k' during elution) of about 30 was obtained (Tables 3 and 5). The analytes were eluted to the main column by back-flushing with a buffer containing 18% v/v methanol.

Column switching

A steep pH gradient from pH 5.5 to 2.5 was formed during elution from the anion-exchange gel. A pH of <4 was required for enrichment on ODS-silica and the first portion of the gradient (0.13 ml, pH 5.5–4) was run to waste to avoid band-broadening. About 15% of the HVA and 10% of the 5-HIAA was lost at the beginning of the elution gradient due to pH effects. About 5% of 5-HIAA was lost due to incomplete elution from column 1 to column 2. The recoveries and band-broadening of the system were determined by comparison with direct injections on to the main column (L3 in Fig. 1). The recoveries were in the range of 80–90% (Table 4) but could be increased to over 90% at the expense of increased band-broadening. The column-switching procedures decreased the column efficiency from 59 000 to 57 000 plates m^{-1} in comparison with the main column.

Figure 2
Separation of 3,4-dihydroxyphenylethyleneglycol
(DOPEG) and homovanillic acid (HVA) on
Aminoethyl Bio-Gel P 2 (18 × 3.2 mm). Mobile
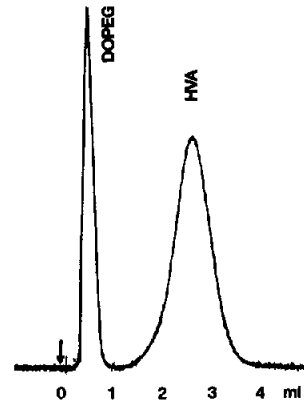phase: 75 mM acetate buffer pH 5.5. Injection
volume: 20 µl.

Table 2
Break-through volumes on the anion-exchange gel

Substance	Total acetate concentration (mM)	V_b* (ml)	V_e† (ml)	k'	N
HVA	100	1.5	2.6	9	56
DOPAC	100	2.1	3.6	13	62
5-HIAA	100	2.7	4.4	17	58
DOPEG	75	0.2	0.5	0.9	—
HVA	75	1.9	3.2	12	75
DOPAC	75	2.8	4.8	18	71
5-HIAA	75	3.4	6.1	24	70

* V_b = break-through volume.
† V_e = elution volume.
N = apparent plate number.

Table 3
Break-through volumes on enrichment column

Substance	V_b (ml)	V_e (ml)	k'	N
DOPAC	5.2	5.8	47	1020
5-HIAA	14	16	130	985
HVA	23	25.3	210	856

Break-through and elution volumes (V_e, V_b) were
determined on 5-µm Nucleosil (20 × 3.8 mm) with
phosphate buffer ($\mu = 0.1$, pH 2.5).

Main separation and detection

The acidic metabolites of the catecholamines and serotonin were readily separated on
the reversed-phase column (Table 5). The separation of 5-HIAA and an unknown
component in plasma improved when 0.5 mM DMOA was added to the mobile phase.
Peaks with retention volumes corresponding to 3,4-dihydroxyphenylpropionic acid

Table 4
Percentage recoveries of DOPAC, 5-HIAA, HVA and 5-HIPA from plasma and buffer solutions

Sample	n	Recovery (% ± S.D.) DOPAC	5-HIAA	HVA	5-HIPA
200 μl acetate buffer pH 5.5	11	81 ± 4	85 ± 4	84 ± 4	85 ± 5
200 μl plasma	10	82 ± 5	84 ± 5	83 ± 1.4	77 ± 1.5

The recoveries from plasma were determined by standard addition and by comparison with direct injections of standard.

Table 5
Characterization of chromatographic peaks by means of capacity factors and detector response ratios

Substance	k'	σ^* (μl)	$\sigma\dagger$ (μl)	Response ratio $CH_2/CH_1\ddagger$ Standard	n	Plasma	n
VMA	0.83	—	—	—		—	
DOPAC	2.7	55	59	0.82	2	—	
5-HIAA	4.0	69	70	0.38	3	0.39	4
DOPPA	5.3	—	—	0.83	2	0.78	8
HVA	6.5	84	92	0.15	3	0.28	6
5-HIPA	7.7	99	103	—		—	
MOPPA	13.3	—	—	0.24	3	0.26	8
IAA	29	—	—	—		—	

$*\dagger$ Standard deviation of peak volume after direct injection (σ^*) and column switching ($\sigma\dagger$).
$\ddagger$ Channel 1 and 2 were operated at $+0.40$ and -0.40 V versus the references respectively.

(DOPPA) and 3-methoxy-4-hydroxyphenylpropionic acid (MOPPA) were detected in plasma and urine (Fig. 3). The response ratio at channels 1 and 2, operated at $+0.40$ and -0.40 V respectively, was used to verify the identity of the compounds by comparison with standards.

The response ratios for 5-HIAA and MOPPA in plasma and standards were similar (Table 5). An unidentified component in plasma, partially resolved from DOPPA, was not reduced at the second electrode. Initially an unknown component with exactly the same retention volume as HVA was detected in plasma. A larger fraction of the compound interfering with HVA was reduced on channel 2 and as a consequence the response ratio corresponding to HVA increased by 87% compared with the standard (Table 5).

The column gave partial resolution of HVA and the interfering peak after several days of conditioning. The peak interfering with HVA was oxidized at a lower potential (Table 6) and the interference was eliminated by coulometric preoxidation at $+0.30$ V on channel 1, followed by detection of HVA at $+0.44$ V on channel 2. The oxidation on channel 1 was also suitable for coulometric detection of 5-HIAA. The final system gave complete separation of 5-HIAA and HVA from the background in CSF, plasma and urine (Figs 4–6).

Figure 3
Chromatogram obtained by injection of a plasma
sample. Injection volume: 200 µl. The conditions
were as described in the text with coulometric
detection at +0.40 V (Ch 1). The compounds were
identified by retention volumes and response ratios
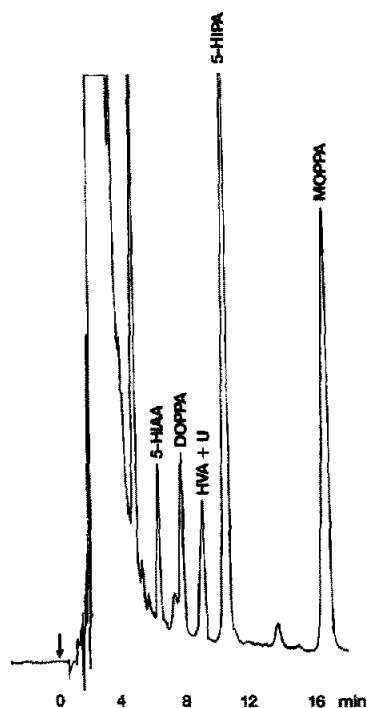(see Table 5). U = unidentified compound.

Table 6
Voltametry of HVA and the HVA-interference peak

Potential (V)	Response 5-HIAA	Unknown	HVA
+0.10	0	3.2	0
+0.20	0	10.8	0
+0.24	31.5	11.6	0
+0.28	53.6	11.9	0
0.32	53.1	11.7	0
0.36	54.0	12.1	3.7
0.40	50.6	12.2	17.1
0.44	51.5	12.1	28.1

Precision and response-stability

The stability and precision of the system were evaluated by repeated injections of
plasma, urine and artificial CSF (Table 7). The intra-assay precision was in the range of
1–6% with a response drift of about 0.5% h^{-1}. The pre-isolation procedure involving
two precolumns was found to be the major source of response drift. Calibration was
performed with four standards with an interval of 15–20 samples. The performance of
the enrichment column deteriorated rapidly after 50–60 plasma injections. The flow
direction of the enrichment column was reversed by change of column inlet and outlet
connections after 45 plasma injections and both precolumns were repacked after 90
plasma injections.

Ch 1.

Figure 4
Chromatograms obtained by injection of a CSF
sample. Injection volume: 200 µl CSF diluted 1:1 in
acetate buffer. The conditions were as described in
the text with coulometric detection at +0.30 V (Ch 1)
and amperometric detection at +0.44 V (Ch 2). 5-
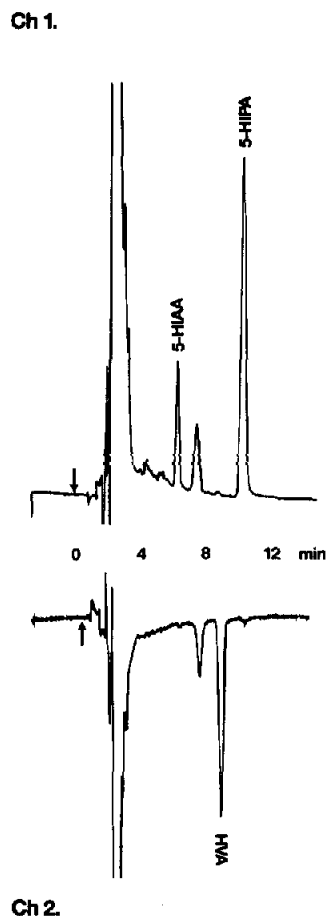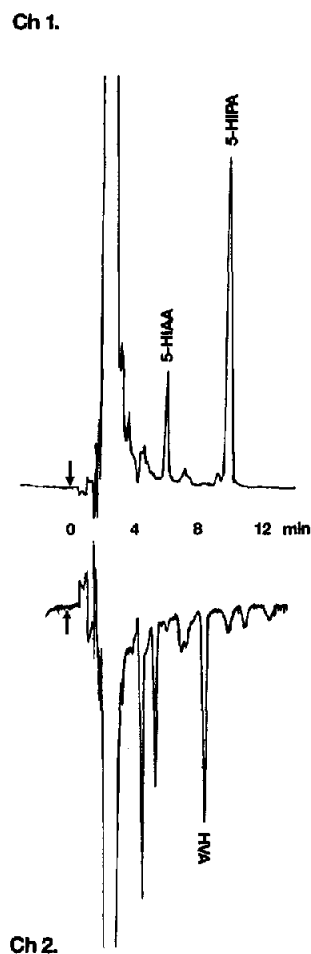HIAA, 58 nmol l^{-1} and HVA, 162 nmol l^{-1}.

Ch 2.

Table 7
Intra-assay precision and system stability

Sample	Substance	Conc.	n	R.S.D. (%)	Response drift (%/10 h)	± S.D.†
CSF	5-HIAA	235 nM	12	2.6	−4.4	±1.2
CSF	HVA	308 nM	12	2.1	−4.7	±1.9
Urine	5-HIAA	11.6 µM	11	1.2	−0.7	±2.3
Urine	HVA	21 µM	11	2.3	−4.8	±2.8
Plasma	5-HIAA	49 nM	23	5.9	+11.5	±2.0
Plasma	HVA	46 nM	23	5.6	−0.30	±2.8
Plasma*	5-HIAA	49 nM	23	5.9	+12.5	±1.7
Plasma*	HVA	46 nM	23	6.1	+1.1	±2.6
Plasma*	5-HIPA	433 nM	23	1.4	+1.4	±0.68

*Calculated from peak heights. Other values calculated from relative peak heights.
†Estimated by linear regression analysis of response or response ratio versus time, with 1–2 injections h^{-1} during 16 h.

Ch 1.

Figure 5
Chromatograms obtained by injection of a plasma
sample. Injection volume: 200 μl. The conditions
were as described in the text with coulometric
detection at +0.30 V (Ch 1) and amperometric
detection at +0.44 V (Ch 2). 5-HIAA, 55.2 nmol l^{-1}
and HVA, 98.1 nmol l^{-1}.

Ch 2.

Accuracy

The method described yields high selectivity for acidic metabolites of biogenic amines
because several of the procedures involved are selective for such compounds; for
instance, isolation on the anion-exchange gel, the reversed phase system and the dual
coulometric/amperometric detection.

Some precautions were undertaken to avoid systematic calibration errors for plasma
samples. The plasma matrix decreased the recovery of the internal standard, compared
with acetate buffer (Table 4), and up to 8% systematic error was obtained after standard
addition and calibration with buffer (Table 8).

5-HIAA and HVA were determined in normal plasma by standard addition and the
plasma was spiked to contain five times the endogenous concentrations. The spiked
plasma was used for calibration and the normal plasma for control samples. Calibration
factors were equal for standards prepared with acetate buffer and artificial CSF diluted
1:1 with acetate buffer.

Figure 6
Chromatograms obtained by injection of a urine
sample. Injection volume: 200 μl urine diluted 1:100
with acetate buffer. The conditions were as described
in the text with coulometric detection at +0.30 V (Ch
1) and amperometric detection at +0.44 V (Ch 2). 5-
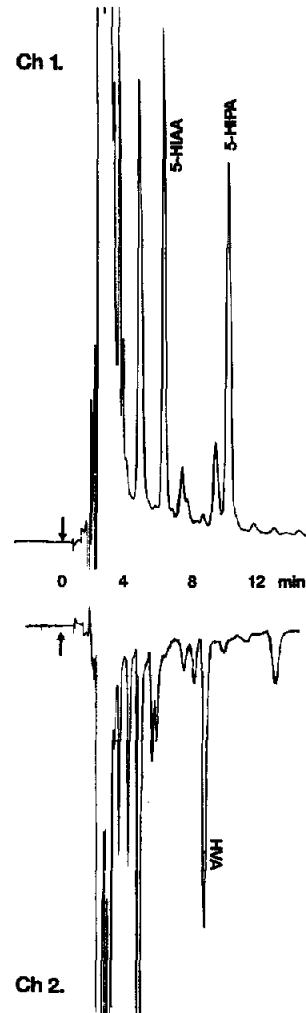HIAA, 10.3 μmol l⁻¹ and HVA, 22.1 μmol l⁻¹.

Table 8
Recoveries after standard addition to plasma and calibration with buffer standard

| Sample | n | Recovery (% ± S.D.)* | | | |
		5-HIAA I.S. method	5-HIAA P.H. method	HVA I.S. method	HVA P.H. method
1	8	106.9 ± 2.6	102.6 ± 2.5	99.7 ± 2	98 ± 5.8
2–15	14	108.3 ± 6.6	96.3 ± 8.4	107.1 ± 8	95.5 ± 9.5

I.S. method = Internal standard method with relative peak heights.
P.H. method = peak height method.
* Correct value: 100%.

Conclusions

Anion-exchange gels based on polyacrylamide gave stable retention volumes with high sample capacity and low non-specific adsorption. The combination of two precolumns, the first for selective isolation and the second for enrichment, made it possible to inject large volumes of biological fluids with a minimum of band-broadening. Coulometric preoxidation facilitated the selective detection of HVA. The instrument gave the sensitivity and stability required for routine analysis of 5-HIAA and HVA in CSF, plasma and urine. Analytical results are obtained within 18 min and about 50 samples per day can be analysed.

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