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CORRELATION BETWEEN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND AUTOMATED FLUORIMETRIC METHODS FOR THE DETERMINATION OF DOPAMINE, 3,4-DIHYDROXYPHENYLACETIC ACID, HOMOVANILLIC ACID AND 5-HYDROXYINDOLEACETIC ACID IN NERVOUS TISSUE AND CEREBROSPINAL FLUID

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SUMMARY

The correlation between automated fluorimetric methods and high-performance liquid chromatography is described for the determination of homovanillic acid and 5-hydroxyindoleacetic acid in cerebrospinal fluid, and for dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid in striata of rat brain. The automated fluorimetric methods for 3,4-dihydroxyphenylacetic acid and homovanillic acid showed a good correlation with the high-performance liquid chromatographic methodology. The fluorimetric determination for dopamine was somewhat less reliable than the high-performance liquid chromatographic assay. The fluorimetric assay for 5-hydroxyindoleacetic acid correlated poorly with the chromatographic method.

INTRODUCTION

Several authors have expressed some doubt about the specificity of the fluorimetric methods used for the analysis of neurotransmitters and metabolites in biological samples [1—3]. Fluorimetric methods can suffer from aspecific fluorescence and quenching. The degree to which these phenomena interfere with the assays depends to a great extent on the quality of the purification procedures. The availability of an automated fluorimetric assay in our laboratory, as well as high-performance liquid chromatographic (HPLC) methodology offered the interesting possibility for a correlation study. Here, we describe the correlation between a fluorimetric method and an HPLC method for the determination of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid (CSF) and for dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA in striata of rat brain.

EXPERIMENTAL

Materials

Materials and their sources were as follows. Dopamine·HCl, 3,4-dihydroxy-phenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid were from Fluka (Buchs, Switzerland). All other chemicals were of analytical reagent grade and were purchased from E. Merck (Darmstadt, G.F.R.). All aqueous solutions were prepared from deionized water distilled in glass. Stock solutions of DA, DOPAC, HVA and 5-HIAA consisted of 100 μg/ml of 0.01 M formic acid and were stored in portions in the freezer (−80°C). Solutions of reference compounds were freshly prepared every week from a portion of the stock solution after appropriate dilution with 0.01 M formic acid.

Animals and dissection

Female albino rats weighing 150—200 g (Wistar; C.D.L., Groningen, The Netherlands) were used. Haloperidol (Serenase; Janssen, Beerse, Belgium) was administered intraperitoneally (1 mg/kg). Rats were killed by decapitation and the brains rapidly removed. After dissection, which was completed within 3 min, the tissue samples were frozen on solid CO_2. Samples were kept at −80°C until assayed.

Isolation procedure on Sephadex G-10

CSF samples (0.5 ml), adjusted to pH 2—3 with 50 μl of formic acid (98%), were applied to Sephadex G-10 columns (5 × 70 mm) prepared in long-size Pasteur pipettes as described previously [4, 5]. Tissue samples were homogenised in 1 ml of 0.1 M perchloric acid (PCA). Following centrifugation (15 min, 4000 g, 4°C) the supernatants were put on the Sephadex columns. At least 80 columns can be handled in one run with the help of automated pipettes. Before use the columns were washed with 3.0 ml of 0.02 M ammonia and 3.0 ml of 0.01 M formic acid. After the samples had passed through the columns, 2.5 ml of 0.01 M formic acid were added. DA was then eluted with 1.0 ml of 0.01 M formic acid followed by 1.5 ml phosphate (0.005 M Na$_2$HPO$_4$·2H$_2$O). DOPAC and HVA were subsequently eluted with 1.1 ml of the phosphate solution. The DOPAC- and HVA-containing fraction was acidified with 50 μl of 6 M formic acid. 5-HIAA was subsequently eluted with 2.5 ml of 0.02 M ammonia. The 5-HIAA-containing fraction was collected in a test tube to which 50 μl of 6 M formic acid were added. HVA and 5-HIAA could be collected in one fraction if, after the 1.5-ml of phosphate wash, the acids were eluted with 2.5 ml of 0.02 M ammonia. The columns were stored in 0.02 M ammonia. The isolation procedure is summarized in Fig. 1.

Chromatography

A Waters Model 6000 A liquid chromatograph was employed in conjunction with an electrochemical detector. The detector was based on the rotating disc electrode principle [6] and it was used in combination with a Bioanalytical Systems potentiostat Type LC-2A. The detector potential was set at 500 mV or 700 mV (for HVA assay) versus a Hg/Hg$_2$Cl$_2$ reference electrode. The
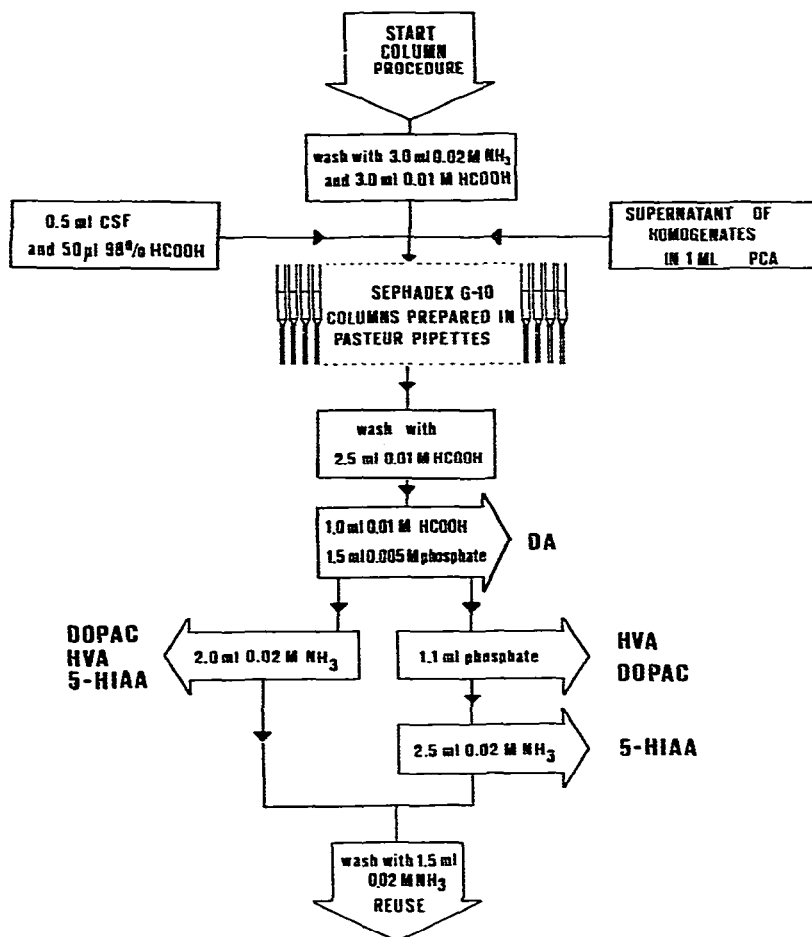
Fig. 1. Flow chart for the isolation procedure.

column (150 × 4.6 mm I.D.) was packed with a slurry of Nucleosil 5C-18 (particle size 5 μm) (Macherey-Nagel, Düren, G.F.R.) reversed-phase material in methanol—carbon tetrachloride (20:80, v/v). The slurry (10%, w/w), degassed in an ultrasonic generator, was pumped into the chromatographic column with a pressure of 40 MPa (the highest possible flow-rate). Columns were washed by passing 200 ml of methanol and further equilibrated with the mobile phase. Analyses were performed at a flow-rate of 60 ml/h at room temperature. The mobile phases consisted of a mixture of McIlvaine buffer (prepared from 0.1 M citric acid and 0.2 M Na$_2$HPO$_4 \cdot$2H$_2$O), 0.1 mM EDTA and methanol. For the assay of DA a mobile phase of pH 5.5 with 3% methanol was used. The eluent for the DOPAC, HVA and 5-HIAA assay was adjusted to pH 3.5 and contained 20% methanol. Injections were made with a high-pressure injection valve (Rheodyne) fitted with a 50-μl or 100-μl sample loop. The concentrations in the biological samples were calculated with the aid of calibration curves obtained after the injection of pure standards. The analytical recoveries of HVA and 5-HIAA were determined by analysing spiked CSF

samples. If the Sephadex eluates had to be preserved until the next day, ascorbic acid (final concentration 10^{-4} M) was added to protect 5-HIAA and the samples were stored at 4°C.

Automated fluorimetry

DA, DOPAC and HVA were assayed according to our previously described method [4, 5]. 5-HIAA was determined as an *o*-phthaldialdehyde derivative [7].

RESULTS AND DISCUSSION

HVA and 5-HIAA in CSF

CSF samples were purified on Sephadex G-10 and subsequently analyzed using HPLC with amperometric detection. Fig. 1 shows that the CSF samples can be purified in two different ways. It is evident from Fig. 2 (B, C) that the collection of HVA and 5-HIAA fractions is not necessary, since the two compounds are well separated under the chromatographic conditions used.

Pooled CSF samples were spiked with 50 ng of HVA and 50 ng of 5-HIAA. The analytical recoveries (± S.D.) of the combined fractions were (data collected from three different experiments): HVA, 94.6 ± 7.2% (n = 12); and 5-HIAA, 95.0 ± 4.9% (n = 12). HVA was determined in a series of CSF samples with the HPLC method and automated fluorimetry. To exclude variation in the methods due to the purification step, eighteen CSF samples were purified on Sephadex G-10 and identical aliquots were used for both the automated fluorimetric method and the HPLC method for HVA. A good correlation was found (r = 0.990, Fig. 3).

The fluorimetric detection for HVA is based on an oxidation-induced dimerization of the HVA molecule, which results in the formation of a very specific fluorophore.

The blank procedure records the background fluorescence of each of the
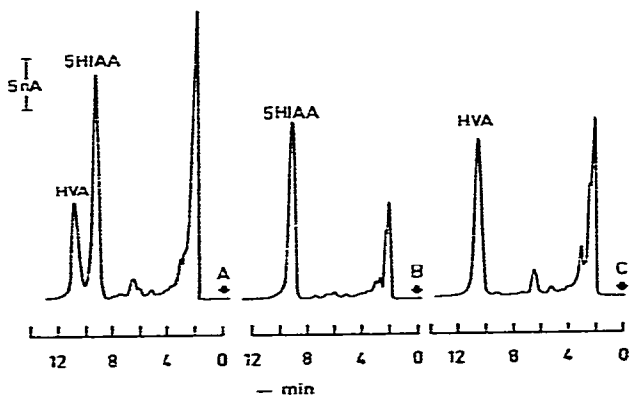

Fig. 2. Chromatograms of a CSF sample purified by two different methods. (1) HVA and 5-HIAA were collected in one fraction of 2.0 ml (A). (2) HVA and 5-HIAA were separated on Sephadex G-10, 5-HIAA was collected in a 2.5-ml fraction (B) and HVA was collected in a 1.1-ml fraction (C). See also Fig. 1.
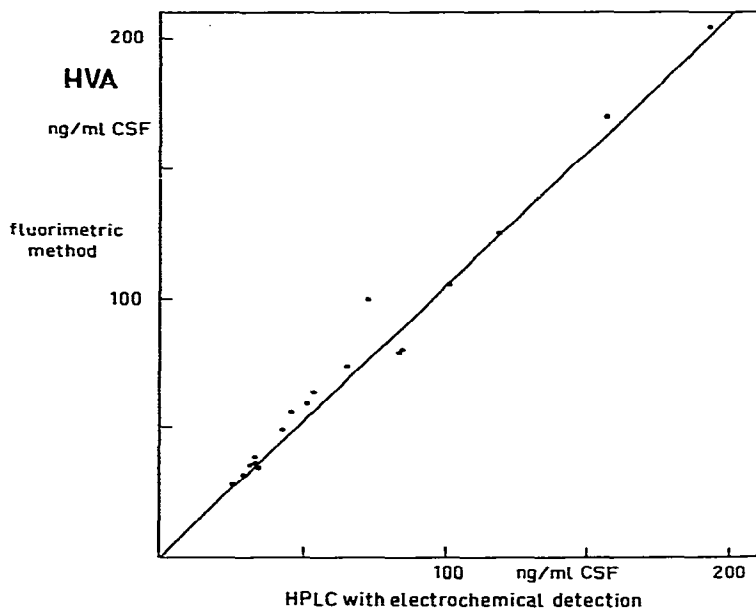
Fig. 3. Correlation between the automated fluorimetric assay and the HPLC method for HVA in CSF samples. The determinations were carried out on identical fractions obtained after purification on Sephadex G-10.

samples individually when oxidation is prevented [4]. The sample clean-up procedure, specific fluorophore formation and appropriate blank procedure resulted in an assay for HVA which is obviously not influenced by aspecific fluorescence or quenching. Muskiet et al. [8] have compared the fluorimetric HVA assay with a mass fragmentographic method and found that for HVA levels below 100 ng/ml the correlation coefficient was somewhat less satisfactory. The authors conclude that "when relatively large changes in the levels of HVA in CSF are to be measured, semiautomated fluorimetric assays may also be sufficiently reliable". The present results are slightly more positive about the automated fluorimetric HVA assay.

Fig. 4 shows the results of the 5-HIAA values obtained by the two methods. The two assays were carried out on identical CSF samples in different laboratories so, unlike in the HVA assay, variations in the isolation procedure are also included in the correlation coefficient. It is obvious that the correlation is poor ($r = 0.773$). Similar results have been obtained by Sjöquist and Johansson [3] for the comparison of a fluorimetric assay and a mass fragmentographic method. The fluorimetric determination used is based on a fluorophore formation between the indole derivative and o-phthaldialdehyde. This reagent is, however, not specific as it reacts with various primary amines and amino acids. Although it cannot be concluded from Fig. 4 which of the two methods is responsible for the poor correlation, we feel that the method based on an efficient chromatographic separation is very likely to be superior to the fluorimetric method. The fact that 5-HIAA values obtained with the fluorimetric method are somewhat higher than the results of the HPLC method
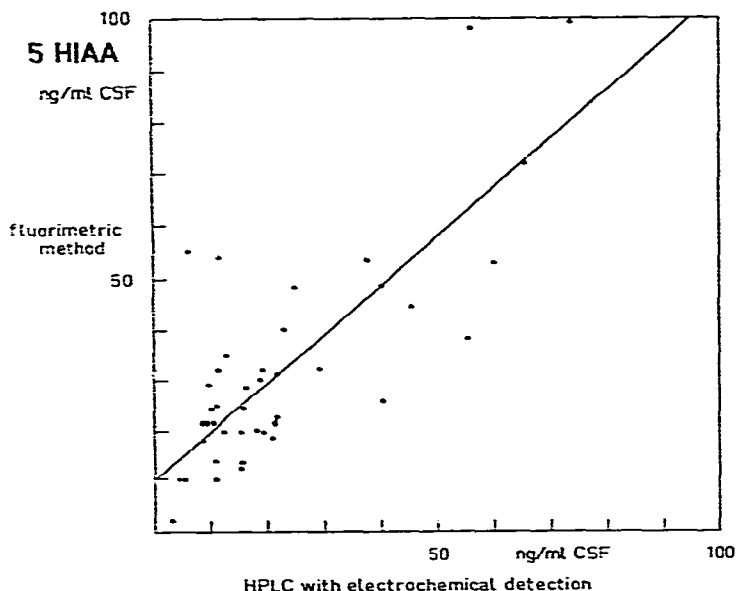
Fig. 4. Correlation between the automated fluorimetric assay and the HPLC method for 5-HIAA in CSF samples. The purification methods for both assays were carried out independently.

(Y-intercept = 10 ng/ml), suggests an aspecific fluorophore formation in the latter method.

DA, DOPAC and HVA in brain tissue

DA, DOPAC and HVA were determined in striata of rat brain. The HPLC assay and the fluorimetric assay were carried out on identical Sephadex fractions, which means that the correlation is only concerned with the detection procedure. Results are given in Figs. 5—7 and summarized in Table I.

Fig. 5 shows the correlation graph for DA. The HPLC method resulted in higher concentrations, and there was a considerable scatter of points around the calculated regression line. The fluorimetric assay for DA was based on fluorophore formation by reaction with ethylenediamine. The aspecific fluorescence of this method, which was estimated by recording the fluorescence of nervous tissue samples obtained from non-dopaminergic brain areas such as the cerebellum, is usually very low (less than 5% of the DA-induced fluorescence in the striatum [5]). Quenching of the fluorescence is a likely explanation for the lower DA concentrations found with the fluorimetric method. This is supported by the fact that the analytical recovery (300 ng of DA added to cerebellar tissue on different days) is more complete for the HPLC method (87.4 ± 6.2% (S.D.), n = 34) than for the fluorimetric method (73.4 ± 6.3% (S.D.), n = 31).

A good correlation was found for DOPAC and HVA for the two analytical methods (Figs. 6 and 7). Ethylenediamine is used to induce a DOPAC-specific fluorescence in a similar manner to the fluorimetric assay of DA. Unlike the DA assay there is no evidence for quenching. In comparison with DA, the
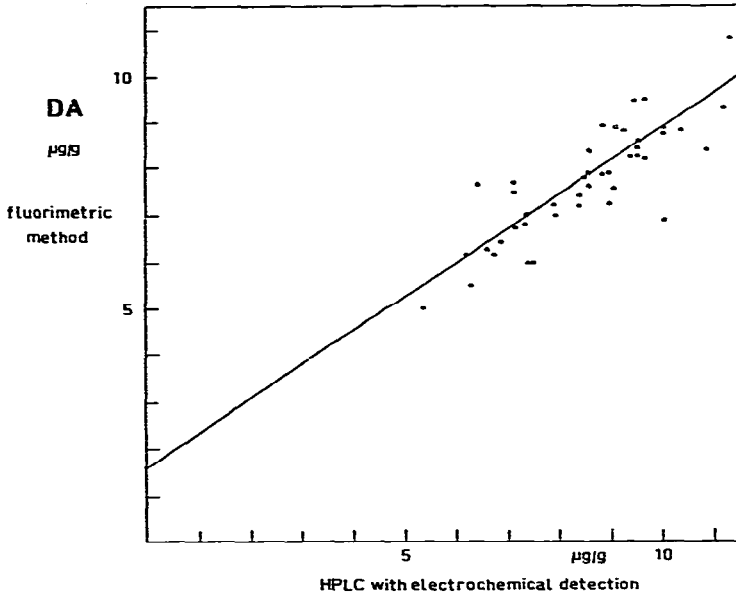
Fig. 5. Correlation between the automated fluorimetric method and the HPLC method for DA in striata of rats. The determinations were carried out on identical fractions obtained from the Sephadex G-10 procedure.
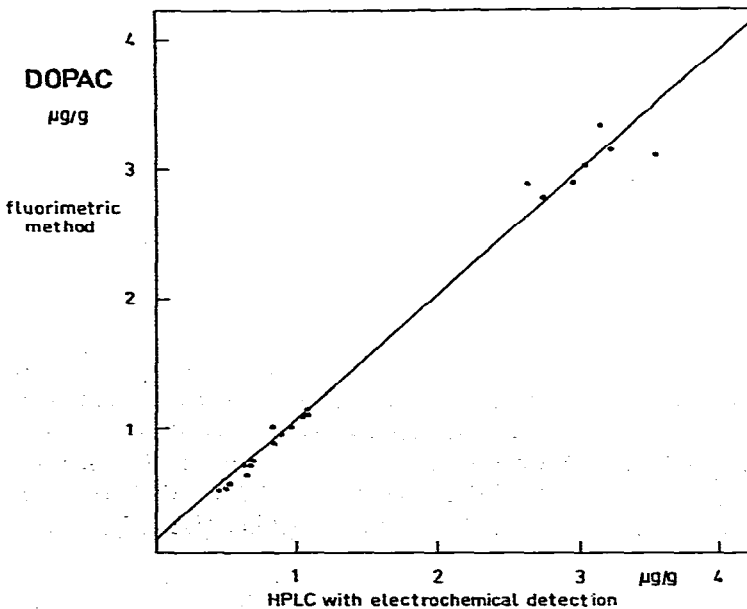
Fig. 6. Correlation between the automated fluorimetric method and the HPLC method for DOPAC in striata of rats. The determinations were carried out on identical fractions obtained from the Sephadex G-10 procedure. Seven samples were obtained from haloperidol-treated rats which causes an increase in DOPAC levels.
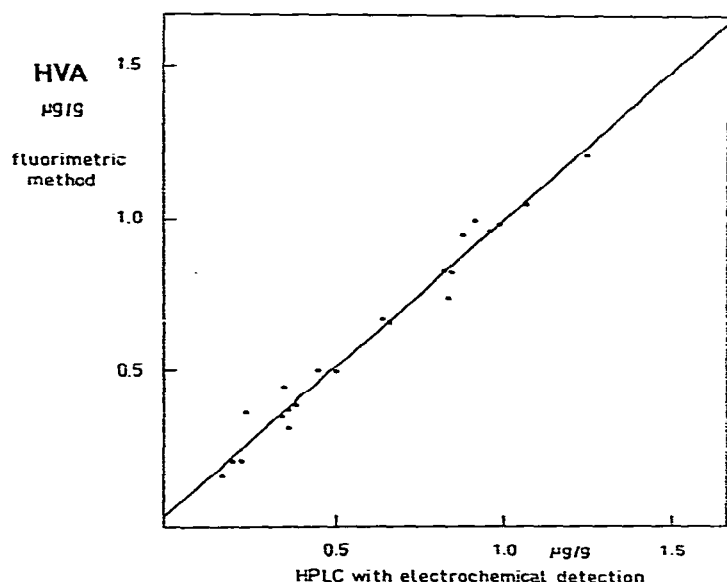
Fig. 7. Correlation between the automated fluorimetric assay and the HPLC method for HVA in striata of rats. The determinations were carried out on identical fractions obtained from the Sephadex G-10 procedure. Nine samples were obtained from haloperidol-treated rats which causes an increase in HVA levels.

TABLE I

SUMMARY OF THE CORRELATIONS FOUND FOR VARIOUS ASSAYS

Method	r	Slope	Intercept
HVA in CSF (Fig. 3)	0.990	1.066	0.45
5-HIAA in CSF including the Sephadex G-10			
procedure (Fig. 4)	0.773	0.945	10.0
DA in striata (Fig. 5)	0.849	0.725	1.61
DOPAC in striata (Fig. 6)	0.993	0.973	0.058
HVA in striata (Fig. 7)	0.988	0.965	0.037

purification of DOPAC on Sephadex G-10 is probably more efficient in removing interfering compounds from the tissue samples. The analytical recoveries of DOPAC and HVA (30 ng added to cerebellar tissue) were monitored weekly in our laboratory, the results ($\pm$ S.D.) were similar for the two methods. HVA (fluorimetric), $76.7 \pm 9.3\%$ ($n = 24$); and HVA (HPLC), $76.1 \pm 7.5\%$ ($n = 25$). DOPAC (fluorimetric), $82.2 \pm 10.7\%$ ($n = 25$); and DOPAC (HPLC), $81.4 \pm 6.2\%$ ($n = 23$).

CONCLUSIONS

In conclusion, semiautomated fluorimetric methods for DOPAC and HVA in brain tissue and HVA in CSF showed a good correlation with HPLC methods

based on amperometric detection. The fluorimetric determination of DA (ethylenediamine method) in striatal samples appeared somewhat less reliable than the HPLC method, probably due to fluorescence quenching. Although the fluorimetric assay of DA is useful for quantitation of DA in striatal samples, lower recoveries and more experimental variation are to be expected. The fluorimetric determination of 5-HIAA in CSF (*o*-phthaldialdehyde method) is not very reliable, as it correlated very poorly with the chromatographic method. Determinations of 5-HIAA in CSF have often been performed in biological psychiatric research [9, 10]. The present data imply that the results of these studies should be interpreted with care when fluorimetric methods are used.

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