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Evaluation of sample work-up methods and internal standards for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in brain by HPLC with electrochemical detection*

A. DUCHATEAU,† H. MARTENS,‡ H. TEUCHY,† A. SCHOUPS§ and M. CLAEYS‖**

Departments of † Biochemistry
‡ Chemistry, University of Limburg, B-3610 Diepenbeek
Departments of § Medicine
‖ Pharmaceutical Sciences, University of Antwerp (UIA), B-2610 Wilrijk, Belgium

Abstract: A high-performance liquid chromatographic method with electrochemical detection is described for the determination of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in brain. Results of studies on sample work-up methods and on the use of various internal standards are reported. The reproducibility of determination of 5-HT and 5-HIAA in two regions of rabbit brain has been evaluated.

Keywords: *5-Hydroxytryptamine; 5-hydroxyindoleacetic acid; reversed-phase high-performance liquid chromatography; electrochemical detection.*

Introduction

A variety of methods has been reported for the simultaneous determination of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in brain homogenates by HPLC with electrochemical detection (ED). Simplicity and sensitivity are important features of the HPLC-ED technique. However, critical parameters affecting accuracy and precision, such as the stability of 5-HT and 5-HIAA in aqueous solution and the selection of a suitable internal standard, have often been ignored.

5-HT and 5-HIAA are subject to considerable degradation in acid media [1–3]. Addition of antioxidants to overcome the instability can even decrease the stability of the 5-hydroxyindoles [4], depending on the type of antioxidant used. In many investigations a structural analogue of the compounds studied has been used in the assay to compensate for losses during sample work-up. In case the compounds to be determined are unstable, the structural analogue has to possess virtually the same stability characteristics as those

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**To whom correspondence should be addressed.

of the compounds under investigation. In the present study, the stability of the 5-hydroxyindoles has been evaluated in perchloric acid solutions and in the HPLC eluent. With the aim of selecting a suitable internal standard, four structural analogues of 5-HT and 5-HIAA have been compared: N_ω-methyl-5-hydroxytryptamine (5-MHT); 5-hydroxyindolecarboxylic acid (5-HICA); 5-hydroxyindole (5-HI); and 5-hydroxyindolepropionic acid (5-HIPA). The behaviour of the internal standards in both standard solutions and biological samples has been evaluated.

Experimental

Chemicals

Chemicals were obtained from the following sources: 5-hydroxytryptamine creatinine sulphate, 5-hydroxyindoleacetic acid, N_ω-methyl-5-hydroxytryptamine oxalate (5-MHT), 5-hydroxyindolecarboxylic acid (5-HICA), 5-hydroxyindole (5-HI), 5-hydroxyindolepropionic acid (5-HIPA), homovanillic acid (HVA) and sodium-1-heptane sulphonic acid from Janssen Chimica (Beerse, Belgium); all other chemicals were obtained from Merck (Darmstadt, FRG).

Apparatus

The chromatographic system comprised a model M45 pump (Waters Assoc., Milford, MA, USA), a six-port injection valve (Valco, Houston, TX, USA), and a 3-μm Spherisorb ODS column (70 × 4.6 mm i.d., Alltech Europe, Eke, Belgium). For electrochemical detection, a LC-4B detector (Bioanalytical Systems, West Lafayette, IN, USA), equipped with a glassy carbon working electrode operated at 0.50 V versus a Ag–AgCl reference electrode, was used. The mobile phase comprised 70 mM phosphate buffer (pH 4.35), 1 mM Na_2EDTA, 0.5 mM heptane sulphonate and 9% (v/v) methanol. The eluent flow-rate was 1.9 ml min^{-1}.

Procedure

Brain regions were homogenized in either perchloric acid or the HPLC eluent. Homogenization was performed on ice using a Duall tissue grinder (Kontes, Vineland, NJ, USA). With perchloric acid for homogenization, the subsequent sample work-up was performed according to Morier and Rips [5]. After homogenization in HPLC eluent, the homogenate was centrifuged at 25,000 **g** and 4°C for 10 min. The supernatant was ultrafiltered using a micropartition system (Amicon, Danvers, MA, USA). The ultrafiltrate was injected directly on the HPLC column.

Results and Discussion

It is well known that in aqueous solution 5-hydroxyindoles can undergo temperature- and time-dependent degradation due to oxidation; the rate can further be enhanced in the presence of metal ions [1–4, 6–12]. The use of an internal standard for analytical measurement is generally adopted in order to correct for losses during sample work-up. However, when the compounds to be measured are unstable, the internal standard has to possess the same stability characteristics as the compounds studied in order to fulfil its purpose. In this study the stability of the 5-hydroxyindoles has been expressed in terms of the peak-height ratio of the compound studied (5-HT, 5-HIAA) versus the internal standard. A change of the ratio gives information about the stability behaviour of the

internal standard in comparison with the compounds studied or vice versa. For instance an increase in the ratio may correspond with an unstable internal standard, or both the compound under investigation and the internal standard are unstable with a higher rate of degradation for the internal standard. The chemical structures of 5-HT, 5-HIAA and of the internal standards, 5-HI, 5-HIPA, 5-HICA and 5-MHT, are given in Fig. 1.

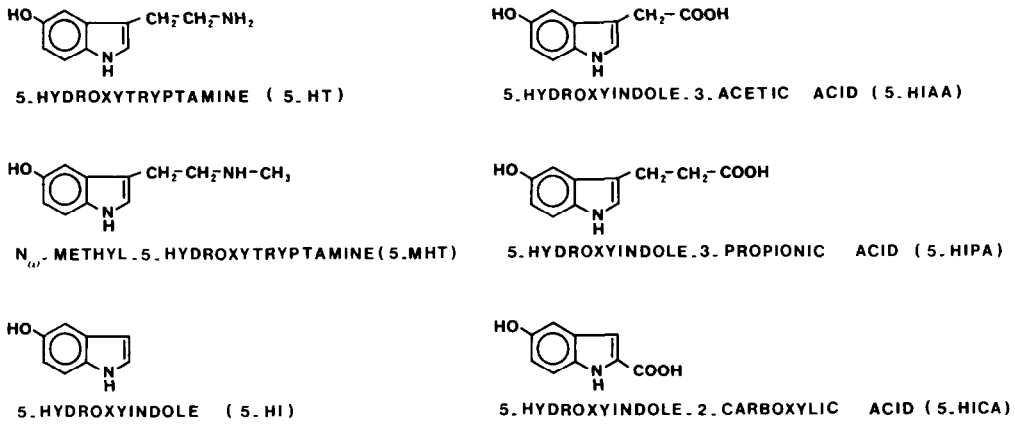

Figure 1
Structures of 5-HT and 5-HIAA and of structural analogues evaluated as internal standards for use in HPLC-ED assays.

Short-term evaluation of the stability of 5-hydroxyindoles

The results obtained for a short-term evaluation (6 h) of the stability of 5-HT and 5-HIAA and the internal standards, 5-HI and 5-HIPA, in different media are presented in Fig. 2.

The data given in Fig. 2 indicate that neither 5-HI nor 5-HIPA can be used as the internal standard to compensate for the losses of 5-HT and 5-HIAA in perchloric acid. The constant ratio found for 5-HT and 5-HIAA in biological samples when using 5-HIPA as the internal standard (Fig. 2A), can possibly be explained by the presence of endogenous antioxidants, e.g. glutathione, which protect the compounds from oxidation to the same extent. Addition of antioxidants such as bisulphite and/or metal chelating agents such as EDTA is a procedure used in many laboratories to suppress oxidative degradation of 5-hydroxyindoles. In this study, it has been found that the addition of bisulphite and EDTA to the perchloric acid solution does not eliminate the oxidative degradation of the internal standard 5-HI in biological samples (Fig. 2B). The reason for the decrease in the 5-HT:5-HI ratio and the 5-HIAA:5-HI ratio in perchloric acid solution containing potassium metabisulphite and disodium EDTA is not clear since the decrease indicates that in this medium, 5-HI is more stable than 5-HT and 5-HIAA. The increased degradation of 5-HI in comparison with 5-HIPA in perchloric acid solutions of biological samples, with and without antioxidants, can readily be explained by the chemical structure of these compounds. Because of the absence of an electron-withdrawing group on the indole ring, the 5-hydroxy group of 5-HI will be very susceptible to oxidation, in contrast with 5-HIPA where the 5-hydroxy group is less electron-dense because of the -I effect of the carboxylic acid function on the indole ring. In the HPLC eluent both 5-HIPA and 5-HI can be used as internal standards over a

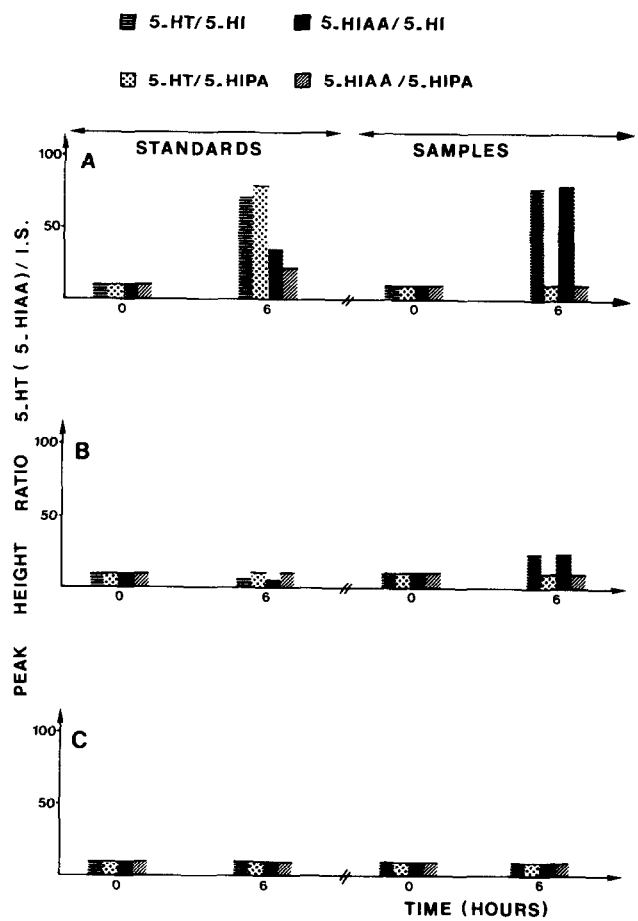

Figure 2
Stability of 5-HT, 5-HIAA and the internal standards 5-HI and 5-HIPA in: 0.4 M aqueous perchloric acid (A); 0.4 M aqueous perchloric acid containing 0.1% (w/v) Na_2EDTA and 0.1% (w/v) $K_2S_2O_5$ (B); and the HPLC eluent (C). The stability has been evaluated for both standard solutions and biological samples. Results are expressed as the peak-height ratios determined for thawed samples and freshly prepared standards and for the same solutions after the storage times indicated in the figure.

period of 6 h when samples and standard solutions are stored at −20°C. Since time and solvent are required for column priming and equilibration, it is often advantageous and convenient to accumulate a number of samples prior to chromatographic analysis. Therefore, internal standards have also been evaluated for use when samples are stored for several days.

Long-term evaluation of the stability of 5-hydroxyindoles

In addition to 5-HI and 5-HIPA, 5-HICA and 5-MHT have also been included as internal standards in samples and standard solutions for the long-term (4 days) evaluation of the stability of 5-hydroxyindoles. For 5-HI, an increase was found in samples for both the 5-HT and 5-HIAA ratios (Fig. 3), whereas the 5-HT:5-HI and 5-HIAA:5-HI ratios remained constant in standard solutions (results not shown). For 5-

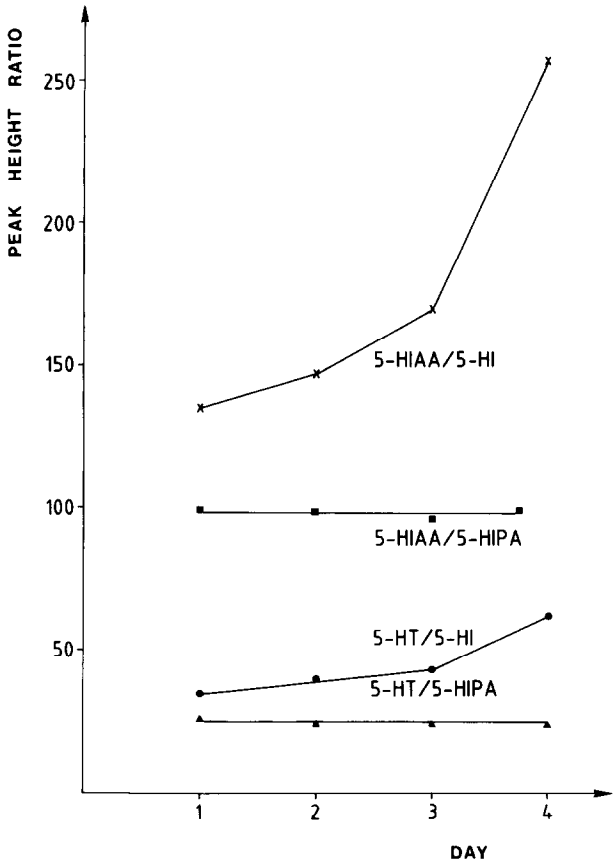

Figure 3
Stability of 5-HT, 5-HIAA and the internal standards 5-HI and 5-HIPA in the HPLC eluent for a biological sample. The sample was assayed in triplicate on each day and the mean of the calculated ratios is given in the figure.

HIPA, 5-MHT and 5-HICA, the 5-HT and 5-HIAA ratios remained constant for 4 days at −20°C for both samples and standard solutions. Because of the instability of 5-HI in the HPLC eluent over the storage period studied, this internal standard was not evaluated as an internal standard for the determination of 5-HT and 5-HIAA. A possible explanation for the instability of 5-HI could be the presence of oxidative enzymes in the biological samples.

Reproducibility of the determination of 5-HT and 5-HIAA in brain samples

A further comparison of 5-HICA, 5-HIPA and 5-MHT as internal standards has been carried out by quantifying the 5-HT and 5-HIAA content of a rat brain sample by means of each of these internal standards. This experiment revealed that the 5-HT and 5-HIAA concentrations determined using 5-MHT as internal standard were not significantly different from the values obtained with 5-HIPA as internal standard (results not shown). However, the 5-HT and 5-HIAA values determined by means of 5-HICA as internal standard were significantly different from the corresponding values obtained with 5-MHT. Factors such as instability and co-elution, however, cannot explain this

discrepancy since 5-HICA was found to be stable in the HPLC eluent and since co-elution of 5-HICA was not observed with an endogenous substance. The higher 5-HT and 5-HIAA contents found when 5-HICA was used as the internal standard may indicate that there is a selective loss of 5-HICA during sample work-up, possibly due to adsorption on to cellular components. On the basis of these results, 5-HICA was considered to be less suitable as the internal standard in the assay of 5-HT and 5-HIAA. As the same results were obtained with 5-HIPA or 5-MHT for the determination of 5-HT and 5-HIAA, the chromatogram could be simplified by using only one of these two internal standards. Representative chromatograms obtained for the determination of endogenous 5-HT and 5-HIAA in two rabbit brain regions, with 5-MHT as the internal standard are shown in Fig. 4.

The linearity of the calibration graphs for 5-HT and 5-HIAA has been established over the range 0.8–4 ng (5-HT) and 1.2–6 ng (5-HIAA). For 5-HT, as well as for 5-HIAA, linear calibration curves were obtained (5-HT curve: $y = 0.38x - 0.61$, $n = 6$, $r = 0.9988$; 5-HIAA curve: $y = 0.39x - 1.03$, $n = 6$, $r = 0.9990$). The results obtained for the within-day and between-day precision with 5-MHT and 5-HIPA as internal

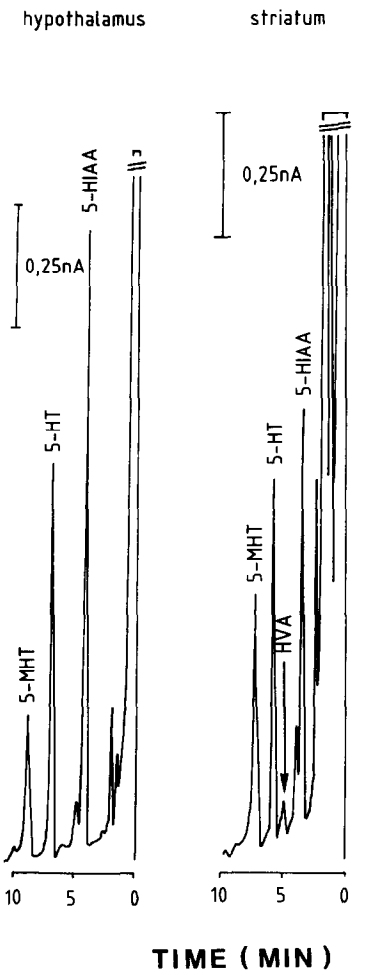

Figure 4
Chromatograms of endogenous 5-HT and 5-HIAA in two regions of rabbit brain with 5-MHT as the internal standard.

Table 1
Within-day and between-day precision, expressed as relative standard deviation (RSD), for the determination of 5-HT and 5-HIAA

Compound measured	5-HT		5-HIAA	
Concentration (ng g^{-1} tissue)	224	198	379	387
Internal standard	5-MHT	5-HIPA	5-MHT	5-HIPA
Within-day RSD ($n = 3$)	0.7%	1.6%	0.6%	0.2%
Between-day RSD ($n = 4$)	1.3%	3.2%	5.2%	5.0%

standards are shown in Table 1 and indicate that the precisions are all better than or near 5%.

Conclusion

The data presented in this study show that the use of the HPLC eluent for homogenizing brain samples and for preparing standard solutions for calibration together with the use of 5-HIPA or 5-MHT as the internal standard, result in an accurate and precise method for the determination of 5-HT and 5-HIAA. The HPLC–ED method has been used to determine the contents of 5-HT and 5-HIAA in regions of rabbit brain [13].

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