Journal of Chromatography, 183 (1980) 483—486
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 634

Note

Comparison of liquid chromatography—electrochemical and gas chromatography—mass spectrometry methods for brain dopamine and serotonin

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(First received March 6th, 1980; revised manuscript received May 15th, 1980)

Among the analytical methods for determination of catecholamines and serotonin (5-HT) in biological tissues, gas chromatography—mass spectrometric (GC—MS) techniques are regarded as the most accurate, specific and sensitive [1,2]. With the advent of the high-performance liquid chromatography with electrochemical detection (LC—EC) (3—5), alternative high-sensitivity assays of these compounds are available. However, whether the accuracy of LC—EC procedures compares favourable with GC—MS techniques has not been established. In the present paper, we report on the direct comparison of LC—EC assay with GC—MS for the simultaneous measurement of dopamine (DA) and 5-HT using rat hypothalamus as a representative brain sample.

EXPERIMENTAL

Procedure

Male Wistar rats (High Oak Ranch, Goodwood, Canada) weighing 300—350 g were housed communally in a temperature (22°C) and light (lights on 08.00—20.00 h) controlled environment for 2 weeks before the experiment. Food and water were given ad libitum. Animals were killed at 13.00 h by decapitation, the brains rapidly removed from the crania and the hypothalami quickly dissected over dry ice using the method described by Holman et al. [6]. The hypothalami were frozen on dry ice and stored at —70°C (less than 24 h). The wet weights of the hypothalami were 40.0 ± 1.9 mg (N = 11).

Each hypothalamus was homogenized in 1 ml of 80% ethanol in a polycarbonate centrifuge tube. Homogenization was performed in an ice bath for 1

0378-4347/80/0000—0000/$02.25

min using a Biosonik ultrasonic probe homogenizer (Bronwill Scientific, Rochester, NY, U.S.A.) at a setting of 5. The homogenate was centrifuged (12,300 *g*) at 4°C for 30 min. Epinine (4 ng) (Aldrich, Milwaukee, WI, U.S.A.), an internal standard for the LC—EC assay, was added to a 200-μl portion of the supernatant, while 20 ng each of α,β-[$^2H_4$]DA and α,β-[$^2H_4$]5-HT (Merck, Sharp & Dohme, Pointe Claire, Canada) were mixed with the remaining 800 μl for GC—MS assay. These supernatant aliquots were then passed through small columns of Amberlite CG-50 as previously reported [5] and the column eluates processed by LC—EC or GC—MS as described below. Tissue pellets were solubilized in 0.3 *M* sodium hydroxide for protein determination [7].

LC—EC and GC—MS assays

The procedure of Warsh et al. [5] was used for the simultaneous LC—EC assay of DA and 5-HT. The Amberlite eluates were evaporated to dryness and the residues reconstituted in 400 μl citrate—acetate buffer (pH 5.2). A 100-μl aliquot was injected into the LC—EC instrument (BAS Model LC-2A equipped with a TL 3 carbon paste electrochemical detector; Bioanalytical Systems, West Lafayette, IN, U.S.A.). Separation of DA and 5-HT was achieved on a 500 × 2.1 mm I.D. glass column packed with Zipax SCX resin (25—37 μm; Dupont Instruments, Wilmington, DE, U.S.A.). The mobile phase consisted of citrate—acetate buffer, pH 5.2 [4] at a flow-rate of 0.5 ml/min. The working electrode potential of the detector was maintained constant at +0.65 V vs. the Ag/AgCl reference electrode.

The GC—MS assays for hypothalamic DA and 5-HT were performed by the methods of Koslow et al. [1] and Warsh et al. [2], respectively, with minor modifications. Briefly, the formic acid—ethanol eluates containing the amines were evaporated to dryness in 3-ml reacti-vials and the residues reacted with 120 μl pentafluoropropionic anhydride (Pierce, Rockford, IL, U.S.A.) in ethyl acetate (5:1, v/v). After removal of the reagents under a stream of nitrogen, the reaction products were reconstituted in 15 μl ethyl acetate and a 2-μl aliquot analyzed by GC—MS using a Finnigan 3200 GC—MS system equipped with multiple programmable ion monitors.

The ion fragments (*m/e*) selected for minitoring were 428 for DA (431 for deuterated-DA) and 438 and 451 for 5-HT (440 and 454 for deuterated 5-HT). The ion fragment ratios at 438/451 for the hypothalamic extracts were identical to those for authentic 5-HT standards, thus providing positive identification for 5-HT; interference from unknown compounds prevented monitoring of a second ion fragment (*m/e* 281) for DA in the tissue samples. For both GC—MS and LC—EC assays, quantitation of DA and 5-HT was performed using calibration curves of the peak height ratios of standard to internal standard vs. the concentration of standard.

Under these conditions, the limits of sensitivity for DA and 5-HT in tissues are 500 pg by the GC—MS method and 100 pg by the LC—EC assay.

RESULTS AND DISCUSSION

Comparison of methods for the determination of brain biogenic amines cannot be done reliably using data derived from different laboratories since a

variety of factors such as differences in animal strain, housing conditions, diet, age and post-mortem handling of tissues may affect the tissue levels of these compounds. Valid direct comparison of methods requires the simultaneous determination of the substance in question in the same tissue sample with each method. This has been achieved in the present report for the comparison of LC—EC with GC—MS methods for DA and 5-HT.

Table I demonstrates that hypothalamic 5-HT concentrations determined by LC—EC are virtually the same as the values obtained by highly specific GC—MS assay (paired $t = 1.65; p > 0.2$). The intra-assay coefficients of variation (C.V.) for the LC—EC assay of 5-HT in replicate (N=6) samples from a pooled standard and tissue extract were 2.8% and 1.4%, respectively. These C.V. values

TABLE I

COMPARISON OF LC—EC AND GC—MS ASSAYS OF RAT HYPOTHALAMIC DA AND 5-HT

Animal No.	DA (ng/mg protein)		Difference (%)	5-HT (ng/mg protein)		Difference (%)
	LC—EC	GC—MS		LC—EC	GC—MS	
1	3.28	3.07	+ 6.84	8.43	7.52	+12.10
2	1.73	1.73	0	7.51	7.58	− 0.92
3	3.09	2.88	+ 7.29	8.15	7.84	+ 3.95
4	3.24	2.89	+12.11	8.35	7.68	+ 8.72
5	3.46	3.11	+11.25	8.23	8.42	− 2.25
6	3.43	2.95	+16.27	7.56	7.57	− 0.13
7	3.01	2.65	+13.58	8.73	7.67	+13.83
8	2.85	2.66	+ 7.14	8.25	7.91	+ 4.29
9	2.97	3.27	− 9.17	9.20	8.99	+ 2.33
10	2.84	2.75	+ 3.27	6.96	7.70	− 9.61
11	2.83	2.49	+13.65	7.64	7.32	+ 4.37
$\bar{X}$	2.98*	2.77	9.14	8.09	7.84	5.68
S.D.	0.47	0.41	4.84	0.63	0.47	4.64

*$p < 0.01$; paired t-test.

agree closely with the values obtained by GC—MS assay (3.8% and 2.2%, respectively). Although the DA concentrations determined by both methods correlated significantly ($r = 0.8$; $p < 0.01$, Pearson Product—Moment correlation) the paired t-test showed the values measured by LC—EC to be slightly (9%) but significantly higher than GC—MS levels (paired $t = 3.17$; $p < 0.01$). However, as for 5-HT, the intra-assay C.V. values for replicate (N=6) samples of DA from a pooled standard and a tissue extract determined by LC—EC (3.1% and 1.6%, respectively) agreed closely with the C.V. values for DA determined by GC—MS (4.3% and 1.2%, respectively).

The basis for the positive bias in DA values determined by LC—EC vs. GC—MS is not clear although several possibilities are likely. Firstly, since we were unable to accurately quantify a second ion fragment in the GC—MS assay of tissue DA, we cannot conclude with complete confidence that the GC—MS assay achieved the highest degree of specificity. However, if the specificity of the GC—MS assay using the ion fragment at m/e 428 were compromised in any

way this would have resulted in elevated values compared to the true tissue concentration. Since the LC—EC values for DA exceed those determined by GC—MS, it is more likely that factors affecting either the specificity and/or accuracy of the LC—EC assay account for the small difference. Although epinine was employed as an internal standard in the LC—EC assay, this compound may be sufficiently different from DA in its chemical characteristics such that it does not provide a complete control for all DA recovery losses in the method. In contrast, deuterated DA used as an internal standard in the GC—MS assay is an ideal internal standard, being only isotopically different from DA. However, epinine appears to be quite satisfactory for use in quantitation of 5-HT by LC—EC.

The higher DA values determined by LC—EC might alternatively arise from a lower specificity of this procedure compared to GC—MS. Since the electrochemical detector is not compound specific, the specificity of the LC—EC procedure is primarily dependent upon the separation and resolution of the compounds chromatographed on the LC column. In the LC—EC assay used here, DA and 5-HT were separated by cation exchange high-performance LC. This column separation may not be optimal for DA determination. In this regard, reversed-phase LC provides superior separations of biogenic amines [8, 9] and may allow even better accuracy in the LC—EC assay.

In summary, quantitation of 5-HT and DA by LC—EC using a cation-exchange column compares very favourably with the GC—MS assay of these compounds although the values obtained for DA are slightly higher. However, the sensitivity of this LC—EC procedure is comparable to that of GC—MS and obviates the need for derivative formation and complex instrumentation in GC—MS assays.

ACKNOWLEDGEMENT

We wish to thank Mrs Susan McNally for her assistance in the preparation of this manuscript.

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