Journal of Chromatography, 430 (1988) 112–117
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4231

Note

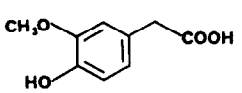
High-performance liquid chromatographic method for measuring homovanillic acid in cerebrospinal fluid using electrochemical detection with internal standardization

GEORGE K. SZABO*,*, HAMID DAVOUDI and RAYMON DURSO

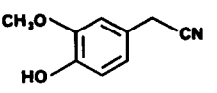
Departments of Neurology and Pharmacology and Experimental Therapeutics, Boston University School of Medicine and Boston Veterans Administration Medical Center, Boston, MA 02130 (U.S.A.)

(First received December 3rd, 1987; revised manuscript received March 28th, 1988)

Homovanillic acid (HVA) (Fig. 1) is a major metabolite of the monoamine neurotransmitter dopamine. Its concentration in cerebrospinal fluid (CSF) has been frequently used to examine abnormalities of dopaminergic transmission in various neuropsychiatric disorders [1–8]. Liquid chromatography (LC) with electrochemical detection (ED) is now the most commonly used technique for quantifying HVA as it is both less expensive and simpler than previous methods employing fluorimetric analysis or gas chromatography–mass spectrometry (GC–MS). Despite its wide-spread use, however, there is little in the literature documenting a high degree of accuracy and precision for LC methods analyzing HVA in CSF, especially at the low concentrations (<10 ng/ml) frequently observed in Parkinson's disease. We report here an accurate and precise isocratic high-performance liquid chromatographic (HPLC) technique for HVA determination



HVA



HVACN

Fig. 1. Chemical structure of HVA and its structural analogue HVACN as the internal standard.

*Present address: Neuropharmacology Laboratory, Department of Neurology (127), V.A. Medical Center, 150 South Huntington Avenue, Boston, MA 02130, U.S.A.

0378-4347/88/$03.50

which employs a structural analogue of HVA as internal standard not previously reported.

EXPERIMENTAL

Instrumentation

This study used a Model 6000 A solvent pump and U6K universal injector both from Waters Assoc. (Milford, MA, U.S.A.). An LC-4B amperometric detector with a glassy carbon working electrode and Ag/AgCl reference electrode from Bioanalytical Systems (West Lafayette, IN, U.S.A.) was used. The chromatographic column consisted of a Partisil-5 ODS-3 (5 μm particle size, 25 cm$\times$4.6 mm I.D.) from Whatman (Clifton, NJ, U.S.A.). For HVA quantitation, a Canon AS-100 computer using software designed by Binary Systems (Newton, MA, U.S.A.) and an analog digital computer interface from Quasitronic (Houston, PA, U.S.A.) was employed.

Reagents and standards

Potassium phosphate (A.C.S. reagent grade) and internal standard, 3,4-hydroxymethoxyphenylacetonitrile (HVACN) (Fig. 1), were obtained from Aldrich (Milwaukee, WI, U.S.A.). Methylene chloride and methanol (HPLC grade) were purchased from Fisher Scientific (Medford, MA, U.S.A.) and HVA from Sigma (St. Louis, MO, U.S.A.). The stock solutions for HVA and HVACN were made by dissolving 1 mg of compound in 10 ml of deionized, redistilled water. They were then frozen at $-80\,^{\circ}C$ in 1-ml aliquots until needed. The working standards for HVA were prepared each day from stock by making serial dilutions to the concentrations required for the standard curve. Working internal standard solution was made daily by diluting from stock to a concentration of 100 ng/ml with deionized, redistilled water.

Chromatographic conditions

The mobile phase was a mixture of 20% (v/v) methanol in 0.075 *M* KH_2PO_4 adjusted to pH 2.5. It was prepared by filtration through a Millipore membrane filter (Millipore, Bedford, MA, U.S.A.), type HA, pore size 0.45 μm. The column temperature was kept ambient. The flow-rate was 1.8 ml/min with the working electrode operated at 1.0 V vs. Ag/AgCl. The detector was set at 1 nA full scale.

Sample preparation

CSF was collected from patients with Parkinson's disease, major depression and Alzheimer's disease, and immediately frozen in 2-ml aliquots. To a 16 mm$\times$150 mm disposable glass test tube capped with inert Tainertops (Fisher Scientific), 500 μl of internal standard solution (HVACN, 100 ng/ml) were added to 500 μl of CSF. The samples were then acidified with approximately 100 μl of 10 *M* hydrochloric acid to a pH less than 1.0. HVA was extracted by adding 5 ml of methylene chloride to samples, shaking them for 10 min on a horizontal shaker and then centrifuging for 2 min to separate the liquid phases. The upper aqueous and interphase layers were then aspirated and discarded. The lower organic layer

was evaporated to dryness under a gentle stream of dry nitrogen in the absence of heat. Subsequently, samples were reconstituted with 200 μl of mobile phase and 100 μl were injected. Under the described procedure, the retention times for HVA and HVACN were 5.0 and 7.0 min, respectively.

Standard solutions (5, 10, 20, 25, 50 and 100 ng/ml) were prepared from HVA stock standard. These standard solutions were taken through the procedure and a linear relationship between standard solution concentrations and peak heights was demonstrated. Quantitation was done by computer, from peak-height ratios of HVA to the internal standard.

RESULTS

Accuracy and precision

In order to evaluate within-day variation we prepared a HVA standard solution with a concentration of 40 ng/ml as a high control point, pooled human CSF with a determined concentration of 28 ng/ml as the midpoint and the CSF pool was diluted to a concentration of 9 ng/ml as the low point. By replicate analysis of these samples we assessed coefficients of variation (C.V.) of 4.0, 2.5 and 4.1% ($n=7$) for low, mid and high points, respectively. Standard curves were set up with each batch of samples to account for any variable that could not be controlled such as changes of ambient temperature and loss of response of the glassy carbon electrode. Linear least-squares regression analysis of the standard curves gave an average r value of 0.997 ($n=3$).

Detector conditions

In this study, an applied potential of 1.0 V was chosen after several higher and lower different voltages were examined. At an applied potential of 0.7 V, a 100-μl injection of HVA and HVACN at 100 ng/ml was undetected. Readable response peaks were first detected at +0.8 V. As demonstrated in a hydrodynamic voltammogram (Fig. 2), both compounds displayed similar response curves with a maximum oxidation potential of +1.0 V.

Mobile phase

After experiments with different methanol concentrations (3–25%), a concentration of 18–22% was found acceptable without causing co-elution of HVA and HVACN. Good resolution between HVA and HVACN and a relatively short total analysis time (9 min) was obtained with a 20% methanol concentration (Fig. 3). Elution times of both internal standard and HVA were found to be significantly affected by mobile phase pH. An increase in pH from 2.5 to 4.5 caused a 13-min increase in retention time of HVACN, while decreasing the elution time of HVA.

Sample preparation

Under acidic conditions (pH 1) the extraction method with methylene chloride was selective for HVA and HVACN and gave absolute recoveries for HVA of 60% and for HVACN of 79% at a concentration of 50 ng/ml. Relative recovery for HVA from standard curve samples ranged from 96.5 to 105.3%. (Table I).

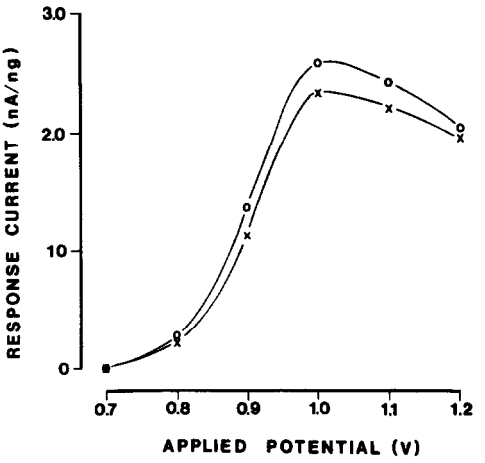


Fig. 2. Hydrodynamic voltammograms of HVA (○) and HVACN (×). The details of chromatographic and detector conditions are described in Experimental and Results.

Fig. 3. Chromatograms demonstrating separation of HVA and HVACN. (A) Aqueous standard solution containing 20 ng/ml HVA and 100 ng/ml HVACN; (B) extracted patient pool CSF containing 28 ng/ml HVA and 100 ng/ml HVACN. Peaks: 1 = HVA; 2 = HVACN.

TABLE I

RELATIVE RECOVERIES

Standard concentration (ng/ml)	Calculated concentration (ng/ml)	Recovery (%)
5.0	4.99	99.8
10.0	10.53	105.3
20.0	19.31	96.5
50.0	50.15	100.3
100.0	101.70	100.2

DISCUSSION

Many LC methodologies for detection of HVA have been published. However, each of these assays had shortcomings that made them unacceptable for our studies. These included: (1) a demonstrable lack of sensitivity and/or precision in the concentration range found in disease states investigated [9–16], (2) no use of an internal standard [9,10,12,16–18], (3) internal standardization utilizing metabolites to be measured [9,11,14,16,18], (4) complex sample preparation [10,12,16,17,19,20] and (5) requirement for expensive special equipment such as multiple-electrode ED [9,12,17,19,21,22].

Our methodology addressed all of these issues. We were able, through employment of HVACN as an internal standard, to obtain accuracy and precision with coefficients of variation less than 5% even for lower concentration ranges of HVA (9 ng/ml). This assay, involving a simple one-step extraction and the use of only elementary LC–ED apparatus, resulted in a fast and sensitive method (levels of 5 ng/ml HVA could be detected) in which no interfering substances from CSF were present.

Using a one-electrode system we could not measure low concentrations of HVA simultaneously with other neurotransmitter metabolites. This finding likely relates to the relatively low detector response of HVA. Mayer and Shoup [12] also noted this problem when assaying concurrently norepinephrine, epinephrine, dopamine, serotonin, 3,4-dihydroxyphenylacetic acid, 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid (5-HIAA) and HVA. They reported that all compounds examined, except HVA, were oxidized under "diffusion-limiting conditions" at a potential of 800 mV. We found at this potential that low-level HVA quantitation by peak height or area measurement from direct injections of unextracted CSF samples was non-reproducible. Increasing injection volume augmented peak height and area but reduced chromatographic resolution and did not improve precision. We, therefore, needed to employ applied potentials above 800 mV and found after voltammogram determinations that 1 V was optimal for detection of HVA and internal standard. Use of this higher potential, however, elicited additional interfering chromatographic peaks with HVA that were not present at the lower potentials and thus an extraction method isolating HVA and internal standard was required.

Whether to remove endogenous interferences or improve sensitivity, other studies [13,17,19,20,23,24] have also employed extraction techniques for measuring HVA in CSF, brain tissue and other physiological fluids. We chose a liquid–liquid extraction to isolate HVA and internal standard. Use of ethyl acetate and diethyl ether as extractants were not suitable because of their lack of selectivity. In addition to HVA, ethyl acetate and diethyl ether tended to extract 5-HIAA and other phenolic acids present in CSF. These latter compounds, when concentrated, interfered significantly with the chromatographic resolution of HVA. Furthermore, neither ethyl acetate nor diethyl ether extracted HVACN reproducibly. Diethyl ether, primarily due to its extreme volatility, had an added disadvantage of requiring special precautions for its use. We eventually found one extractant to be free of all these disadvantages: methylene chloride isolated HVA and HVACN in a selective and reproducible manner.

Our choice of HVACN as an internal standard was based on its similar structure to HVA as well as its ability to behave electrochemically and chromatographically like HVA. It is our belief that accurate determination of this metabolite using HPLC necessitates the use of a good internal standard. This is in agreement with Van Bockstaele et al. [11] who stated "In order to determine quantitatively the metabolites in CSF various procedures can be used. Precise and accurate results will be only obtained by using an internal standard method". We find that manipulation of samples (e.g. extraction) as well as gradual loss of detector response as a function of time and applied voltage are clear sources of error requiring use of an internal standard. This response loss is inherent to electrochemical detectors commonly in use today and in our experience can occur over a relatively short period of time (as little as 5–6 h).

Instead of using a single compound as an internal standard, a number of published methods [10,11,13,16,18] have utilized a procedure, the standard addition technique, whereby samples to be analyzed are spiked at some point during their preparation with known amounts of metabolites being assayed. We did not attempt to examine this methodology and felt that spiking samples with concentrations greater than that of the original sample value could not give quantitatively accurate and reproducible measurements. This view was supported by the lack of precision data presented in these papers.

REFERENCES

1 C.M. Banki, J. Neurochem., 28 (1977) 255.
2 S.R. Bareggi, M. Franeschi, L. Bonini, L. Zecca and S. Smirne, Arch. Neurol., 39 (1982) 709.
3 R.H. Gerner, L. Fairbanks and G.M. Anderson., Am. J. Psychiatry, 141 (1984) 1533.
4 C.J. Gibson, M. Loge and J.H. Growson, Arch. Neurol., 42 (1985) 489.
5 F.K. Goodwin, R.M. Post, D.L Dunner and E.K. Gordon, Am. J. Psychiatry, 130 (1973) 73.
6 R. Mayeux, Y. Stern, M. Sano, L. Cote and J.B.W. Williams, Neurology, 37 (1987) 1130.
7 J.J. Mann, M. Stanley, R.D. Kaplan, J. Sweeny and A. Neophytides, J. Neurol. Neurosurg. Psychiatry, 46 (1983) 905.
8 F. Papeschi and D.G. McClure, Arch. Gen. Psychiatry, 25 (1971) 354.
9 G.M. Anderson, M.B. Bowers, R.H. Roth, J.C. Young, C.C. Hrbek and D.J. Cohen, J. Chromatogr., 277 (1983) 282.
10 A.J. Cross and M.H. Joseph, Life Sci., 28 (1981) 499.
11 M.V. Van Bockstaele, L. Dillen, M. Claeys and W.P. Depotter, J. Chromatogr., 275 (1983) 11.
12 G. Mayer and R. Shoup, J. Chromatogr., 255 (1983) 533.
13 E. Gerlo and R. Malfait, J. Chromatogr., 343 (1985) 9.
14 M.A. Javors, C.L. Bowden and J.W. Maas, J. Chromatogr., 336 (1984) 259.
15 M.H. Joseph, B.V. Kadam and D. Risby, J. Chromatogr., 226 (1981) 361.
16 B.H.C. Westerink, J. Chromatogr., 233 (1982) 69.
17 A.M. Krstulović, L. Bertaini-Dziedzic, S. Bautista-Cerqueira and S.E. Gitlow, J. Chromatogr., 227 (1982) 379.
18 L. Elrod and G.L. Mayer, J. Liq. Chromatogr., 8 (1985) 1817.
19 A. Minegishi and T. Ishizaki, J. Chromatogr., 308 (1984) 55.
20 F. Hefti, Life Sci., 25 (1979) 775.
21 W.R. Matson, P. Langlais, L. Volicer, P.H. Gamache, E. Bird and K.A. Mark, Clin. Chem., 30 (1984) 1477
22 G.M Anderson and J.G. Young, Life Sci., 28 (1981) 507.
23 W.H. Chang, M. Scheinin, R.S. Burns and M. Linnolia, Acta Pharmacol. Toxicol., 53 (1983) 275.
24 P.T. Kissinger, C.S.Bruntlett and R.E. Shoup, Life Sci., 28 (1981) 455.