Journal of Chromatography, 320 (1985) 414–420
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,372

Note

High-performance liquid chromatographic determination of hallucinogenic indoleamines with simultaneous UV photometric and voltammetric detection

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(Received November 5th, 1984)

Indoleamine derivatives, which have hallucinogenic properties, are formed in humans as metabolites of tryptophan, especially N-methyltryptamine, N,N,-dimethyltryptamine and N,N-dimethyl-5-hydroxytryptamine, and are contained in some mushrooms (psilocin and psilocybin). As these substances are important in the diagnosis of some psychic diseases, *e.g.*, epilepsy and schizophrenia, and psilocybin and psilocin extracted from mushrooms can be abused, sensitive methods are required for their determination in psychiatry and forensic medicine.

A great variety of analytical methods have been applied to the determination of indole derivatives, but only gas chromatography in combination with mass spectrometry has found wide use (see, *e.g.*, refs. 1–3), as it is sufficiently sensitive and exhibits a high separation efficiency. However, these substances are poorly volatile and therefore derivatization is required, which makes the determination tedious and introduces additional errors. These drawbacks do not arise with high-performance liquid chromatography (HPLC), which has been used to determine indoleamines in body fluids and tissues (*e.g.*, refs. 4–12) and in mushroom extracts[13–18]. Whereas tryptophan metabolites are best separated on non-polar chemically bonded phases[10,12,18], silica gel[13,16,17] and ion exchangers[14] have been used successfully to separate psilocin and psilocybin. In addition to more universal UV photometric detectors, spectrofluorimetric[4–6,14,16,18] and recently electrochemical detectors (*e.g.*, refs. 8–11) have been used. The latter detectors are marked by high sensitivity and selectivity, which are especially important in analyses of biological samples with complicated matrices. For reviews of electrochemical detectors and their application in HPLC see, *e.g.*, refs. 19–21. By connecting a UV photometric and an electrochemical detector series at the column outlet, the resolution of the test mixture is improved in a non-chromatographic manner and identification of the sample components is facilitated.

0021-9673/85/$03.30

In this paper we describe the separation of some hallucinogenic indoleamines with detection with a UV photometric and a voltammetric detector in tandem and the application of the procedure to the determination of these substances in cerebrospinal fluid and some mushrooms.

EXPERIMENTAL

Chemicals

The compounds studied were tryptophan (TRP) (Kodak, U.S.A.); tryptamine (TPA) (Calbiochem, U.S.A.); 5-hydroxytryptamine (5-OH-TPA; serotonin), 5-hydroxy-N,N-dimethyltryptamine (5-OH-DMTPA; bufetonin), 5-hydroxy-N-acetyltryptamine (5-OH-N-acetyl-TPA), N-methyltryptamine (MTPA), N,N-dimethyltryptamine (DMTPA), 5-methoxytryptamine (5-MeO-TPA), 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMTPA) (all from Sigma, U.S.A.); 4-hydroxy-N,N-dimethyltryptamine (psilocin) and 4-phosphoryloxy-N-methyltryptamine (psilocybin) (Merck, F.R.G.). All other chemicals were of analytical-reagent grade from Lachema (Czechoslovakia), except perchloric acid (Merck).

Instrumentation

The liquid chromatograph consisted of a 3B high-pressure pump, a sampling valve with a 10-μl loop, an LC-75 UV photometric detector and a Chromatographics 2 data system (all from Perkin-Elmer, U.S.A.). The UV photometric detector was connected in series with an EDLC voltammetric detector (Laboratorní Přístroje, Czechoslovakia). The chromatographic column used contained Partisil ODS, 10 μm (25 cm × 4.6 mm I.D.) (Pye Unicam, U.K.) or Separon SI C_{18}, 10 μm (25 cm × 4 mm I.D.) (Laboratorní Přístroje). The mobile phase consisted of aqueous citrate–phosphate buffer of pH 3.8 (300 ml 0.1 *M* citric acid and 160 ml of 0.1 *M* NaH_2PO_4) and various amounts of methanol or ethanol. The flow-rate was 1.0 ml/min. All the measurements were carried out at laboratory temperature. The electrode potentials are related to the standard saturated aqueous silver chloride reference electrode (potassium chloride).

Stock solutions of the studied substances were prepared at a concentration of about 10^{-3} *M* in the particular mobile phase and were appropriately diluted with the mobile phase immediately before the measurement.

Sample pre-treatment

Cerebrospinal fluid (CSF). To a 5-ml CSF sample, 70% perchloric acid was added up to a concentration of 7% for the removal of proteins and the mixture was centrifuged for 30 min at 1000 *g*. The pH of the separated supernatant was adjusted to 12 with 45% potassium hydroxide, while cooling in an ice-bath. The potassium perchlorate precipitate formed was separated by centrifugation for 5 min, 1 g of sodium chloride was added to the supernatant and the solution was extracted twice with 6-ml portions of redistilled dichloromethane (15 min shaking). The combined organic extracts were dried by adding 3 g of anhydrous sodium sulphate and centrifuged for 5 min at 1000 *g* to remove the drying agent. The supernatant was transferred into a conical test-tube and the solvent was evaporated *in vacuo* at laboratory temperature. The residue was dissolved in 200 μl of methanol and a 100-μl sample of this solution was injected on to the column.

The same procedure was repeated with a 5-ml CSF sample to which standard methanolic solutions of 5-OH-DMTPA, 5-MeO-TPA, MTPA, DMTPA and 5-MeO-DMTPA were added to give concentrations of these substances of *ca.* 20 ng in the injected sample volume.

Mushrooms[18]. A 300-mg sample of the mushroom *Psilocybe bohemica* Šebek was homogenized and extracted for 24 h on a reciprocating shaker into 30 ml of methanol. The mixture was then filtered, the solvent evaporated *in vacuo* and the residue dissolved in 3.0 ml of methanol. A 10-μl aliquot was injected into the chromatograph. The standard solutions of psilocin and psilocybin contained 1 and 5 μg of the test substances, respectively, in 5 μl of methanol.

RESULTS AND DISCUSSION

The optimal conditions for the separation and the detection of indoleamines were studied. Various stationary phases were tested; the best results were obtained with Partisil ODS and Separon SI C_{18}. Of the mobile phases tested, the best performance was exhibited by an aqueous citrate–phophate buffer mixed with methanol or ethanol in various proportions (with methanol, the column efficiencies for the studied compounds were low; therefore, ethanol was used in subsequent experiments). The dependences of the logarithms of the capacity ratios on the ethanol content in the mobile phase were approximately linear within a range of 10–20% (v/v) (Fig. 1). The optimal separation was obtained with 10% (v/v) ethanol. An example of a separation under these conditions is shown in Fig. 2.

The substances studied adsorb in spectral regions around 220 and 260–290 nm. The optimal wavelength for the detection is 280 nm, where the absorption of the mobile phase is minimal. The substances can further be electrochemically oxidized at carbon electrodes, with half-wave potentials around +0.5 V for hydroxy derivatives and +0.9 to +1.0 V for the other substances. Therefore, a potential of +1.0 V was used for voltammetric detection. Under the optimal conditions for HPLC

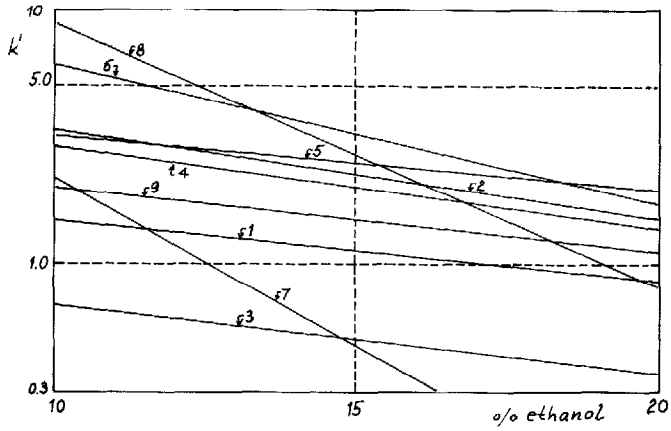

Fig. 1. Dependence of capacity ratio on the ethanol content in the mobile phase. 1, TRP; 2, TPA; 3, 5-OH-TPA; 4, 5-MeO-TPA; 5, MTPA; 6, DMTPA; 7, 5-OH-DMTPA; 8, 5-MeO-DMTPA; 9, 5-OH-N-acetyl-TPA.

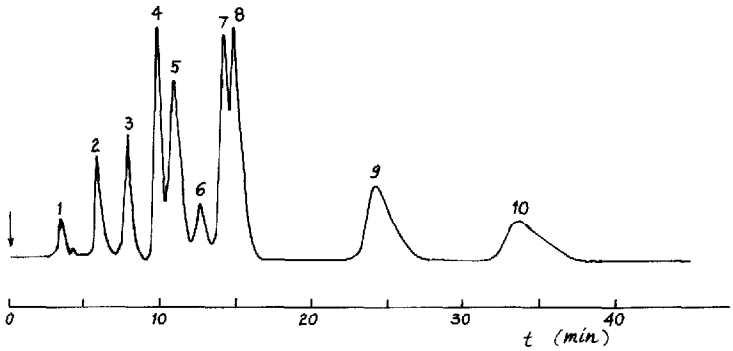

Fig. 2. Chromatogram of a mixture of indoleamines. 1, Methanol; 2, 5-OH-TPA; 3, TRP; 4, 5-OH-N-acetyl-TPA; 5, 5-OH-DMTPA; 6, 5-MeO-TPA; 7, MTPA; 8, TPA; 9, DMTPA; 10, 5-MeO-DMTPA. Electrochemical detector, + 1.0 V (Ag/AgCl). Partisil ODS; mobile phase, 0.1 *M* phosphate buffer + 10% (v/v) ethanol, pH 3.8; flow-rate, 1.0 ml/min.

separation and detection, the calibration graphs and the detection limits were measured and are given in Table I, from which it can be seen that electrochemical detection is more sensitive for all the compounds studied and an especially high sensitivity is obtained for the hydroxy derivatives.

The method was applied to the determination of these compounds in cerebrospinal fluid. The CSF samples available did not contain detectable amounts of the studied substances and thus they were "spiked" with them in order to test the method. The recovery using the procedure described under Experimental was determined by adding known amounts of the standard substances (30 ng of each) to the CSF (obtained from hydrocephalic child patients) and analysing the samples by HPLC with electrochemical detection. The measurements were carried out in quin-

TABLE I

CALIBRATION DATA FOR THE INDOLE DERIVATIVES STUDIED

For conditions, see text. Six parallel determinations, $\alpha = 0.05$.

Substances	*UV photometric detection*			*Voltammetric detection*		
	Correlation coefficient	*Detection limit (ng)*	*Relative standard deviation (%)*	*Correlation coefficient*	*Detection limit (ng)*	*Relative standard deviation (%)*
TRP	0.9933	4.0	2.0	0.9980	3.0	2.4
TPA	0.9991	9.0	2.7	0.9928	6.0	2.5
5-OH-TPA	0.9967	1.7	3.2	0.9986	0.2	3.2
5-MeO-TPA	0.9946	50.0	4.8	0.9996	5.0	2.7
MTPA	0.9917	50.0	4.3	0.9972	5.0	3.7
DMTPA	0.9874	20.0	4.1	0.9897	7.0	3.5
5-OH-DMTPA	0.9989	5.5	3.1	0.9994	0.2	3.5
5-MeO-DMTPA	0.9762	70.0	4.5	0.9823	5.0	3.1
5-OH-N-acetyl-TPA	0.9993	4.4	2.9	0.9995	0.2	2.6
Psilocin	0.9941	80.0	3.0	0.9941	2.0	2.9
Psilocybin	0.9996	93.0	2.8	0.9964	12.0	3.5

TABLE II

REPRODUCIBILITY AND RECOVERY IN THE ANALYSIS OF CSF WITH ELECTROCHEMICAL DETECTION

Amount of each substance taken, 30 ng.

Substance	*Found (%)*	*Relative standard deviation (%)*	*Recovery (%)*
5-OH-TPA	28.4	3.0	94.7
MTPA	27.4	3.7	91.3
5-OH-N-acetyl-TPA	26.3	6.3	87.7
TRP	28.0	5.0	93.3

tuplicate and the results are given in Table II. It follows that in the procedure employed about 10% of the test substances are lost. The relative standard deviation is 3–6%, which is satisfactory. A chromatogram of a CSF sample, with the addition of 20 ng of five hallucinogenic indoleamines, is shown in Fig. 3. As can be seen, the electrochemical detector is sufficiently sensitive to detect the test substances in these amounts, down to the detection limits given in Table I. The UV photometric detector gave no response under these conditions.

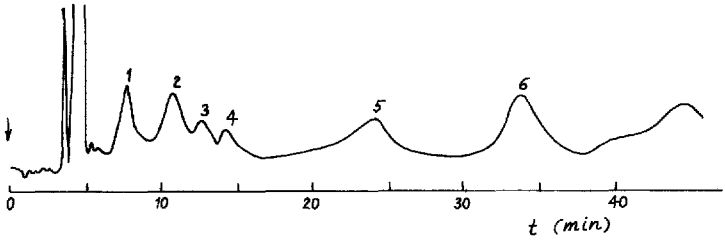

Fig. 3. HPLC analysis of cerebrospinal fluid with electrochemical detection, enriched by the following substances (20 ng of each). 1, TRP; 2, 5-OH-DMTPA; 3, 5-MeO-TPA; 4, MTPA; 5, DMTPA; 6, 5-MeO-DMTPA. Electrochemical detector, +1.0 V (Ag/AgCl). No response was obtained from the UV photometric detector. For other conditions, see Fig. 2.

The other application concerned the determination of hallucinogenic indole amines in mushrooms, where the contents were substantially higher. Under the same experimental conditions as described above, standard solutions of psilocin and psilocybin and samples of the extracts of the mushroom *Psilocybe bohemica* Šebek were analysed, peak heights and areas were evaluated and the contents of these substances were determined from both UV photometric and electrochemical detection signals. The determination from the peak heights is more precise than that from the peak areas and therefore peak-height measurements were subsequently used. The average results and the standard deviations obtained from five parallel determinations at a significance level of $\alpha = 0.05$ are given in Table III. Chromatograms of the mushroom extract, using the two detection techniques, are given in Fig. 4. The detection limits are 93 and 12 ng for psilocybin with UV photometric and voltammetric detection, respectively, the corresponding values for psilocin being 80 and 2 ng.

TABLE III

DETERMINATION OF PSILOCYBIN AND PSILOCIN IN EXTRACT OF *PSILOCYBE BOHEMICA* ŠEBEK USING UV PHOTOMETRIC AND ELECTROCHEMICAL DETECTION

Five parallel determinations from the peak heights, $\alpha = 0.05$.

Compound	*UV photometric detection*		*Electrochemical detection*	
	$\bar{x}$ (%)	*Relative standard deviation (%)*	$\bar{x}$ (%)	*Relative standard deviation (%)*
Psilocybin	0.58	3.02	0.57	2.35
Psilocin	0.058	10.10	0.061	1.37

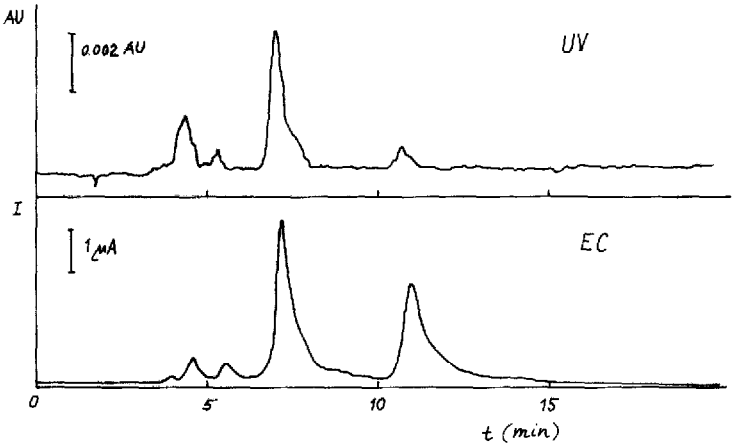

Fig. 4. HPLC analysis of the extract of *Psilocybe bohemica* Šebek with UV photometric and electrochemical detection. Sample, 10 μl. For experimental conditions, see text. Retention times: 7.25 min for psilocybin and 11.0 min for psilocin.

Voltammetric detection seems to be superior to UV detection in determination of the hallucinogenic substances studied, in both CSF and mushrooms. Psilocin is more readily oxidized than psilocybin, owing to the presence of a hydroxy group in the molecule, and its determination with voltammetric detection is much more sensitive than that with UV photometric detection. With CSF, the UV photometric detector gives no signal. The chromatogram of the mushroom extract is simpler when voltammetric detection is used, as other components co-extracted from the mushrooms and absorbing in the UV region do not interfere.

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