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Determination of dopamine and two of its acidic metabolites by high-performance liquid chromatography with electrochemical detection: a retrospective article

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INTRODUCTION

A few decades ago, biogenic amines, such as serotonin and the catecholamines noradrenaline and dopamine, were found to act as neurotransmitters in the central nervous system. It was suggested that abnormalities in these neurotransmitters or transmitter systems may be implicated in mental illnesses such as schizophrenia and affective diseases and also in neurological diseases such as Parkinson's and Huntington's diseases. The relevance of these hypotheses has been studied in a great number of behavioural and biochemical tests both in animals and in human post-mortem material. However, for a long time progress in research in these fields was hampered by the lack of simple methods for the determination of the levels of monoamines and monoamine metabolites in biological tissues and fluids. The methods available were complicated and tedious (radiometric methods), insensitive (fluorimetric methods) or required expensive instrumentation (methods based on mass spectrometry).

In the mid-1970s, an electrochemical detector for high-performance liquid chromatography (HPLC) was developed in Professor Ralph Adams' laboratory. This detector was made commercially available by Dr. P. T. Kissinger and, in combination with progress in HPLC, it became one of the most important tools for transmitter research. All at once, a very selective and efficient analytical technique had become available. As the technique was relatively simple and inexpensive, it resulted in a flood of articles concerning pharmacological and analytical aspects of transmitter research in the late-1970s. Our contribution was a paper [1] describing the direct injection of brain homogenates into a liquid chromatographic system based on a short octadecylsilica column. This paper has since been among the

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twenty most frequently cited articles in *Journal of Chromatography, Biomedical Applications*. The impact of the paper can probably be attributed to the simplicity of the sample preparation and the selectivity and flexibility of the ion-pair chromatographic system. Most important, however, is probably the fact that the paper was published at a time when interest in these kinds of determinations was at its height.

METHODOLOGY AND DISCUSSION

Brief description of method

Rat brain region samples (4–50 mg) were homogenized by sonication in a solution of 0.1 *M* perchloric acid containing an internal standard (epinine). The homogenates were centrifuged and an aliquot of the clear supernatant was injected onto the chromatographic column (100 mm × 3 mm I.D., filled with 5-μm Nucleosil C_{18}). The mobile phase was citrate buffer (pH 4.25)–methanol (92:8, v/v) with the addition of 1.7 m*M* sodium hexyl sulphate. Biogenic monoamines and monoamine metabolites were detected with an LC-2A amperometric detector (Bioanalytical Systems) with a graphite paste electrode operated at +0.60 V. An example of a chromatogram is shown in Fig. 1. Further details are given in the original paper [1].

Sample preparation

In our method we introduced the direct injection of untreated supernatant from brain homogenates onto the chromatographic column for the assay of dopamine and dopamine metabolites. In most methods in the late-1970s, the catecholic compounds were isolated from the matrix by homogenizing the brain, or parts thereof, in perchloric acid and, after centrifugation, the supernatant was transferred into a vial with alumina in

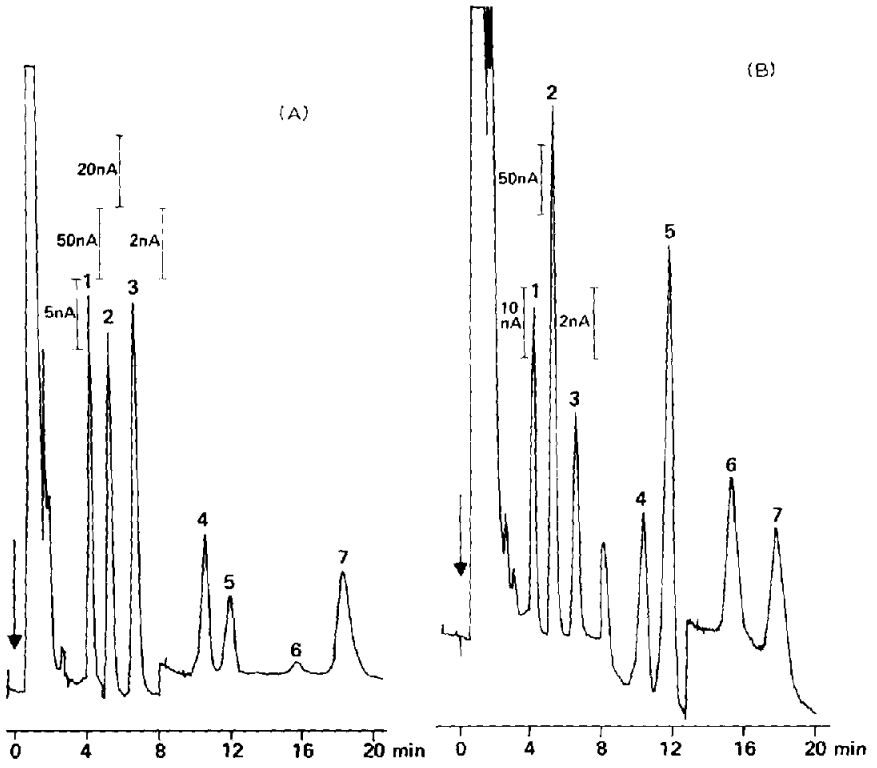

Fig. 1. Chromatograms of rat striata: effect of detector potential, (A) 0.60 V and (B) 0.80 V. Support, Nucleosil C_{18}, 5 μm; mobile phase citrate buffer (pH 4.25)–methanol (92:8, v/v) containing sodium hexyl sulphate (1.7 m*M*). Peaks: 1 = 3,4-dihydroxyphenylacetic acid (7.5 pmol); 2 = dopamine (99 pmol); 3 = epinine (50 pmol); 4 = 5-hydroxyindoleacetic acid; 5 = homovanillic acid (7.8 pmol); 6 = 3-methoxytyramine; 7 = serotonin. From ref. 1.

alkaline buffer. After some washing steps, the alumina was soaked with perchloric acid and centrifuged and thereafter the supernatant was filtered and injected onto the chromatographic column (*e.g.*, ref. 2). Alternatives to the alumina adsorption–desorption were liquid–liquid extraction [3] or the use of a cation-exchange column [4].

The alumina adsorption–desorption gave an efficient clean-up of the supernatant but had some drawbacks. The recoveries of the catecholamines were by no means quantitative, and the procedure was tedious. To us, however, the most serious drawback was that all acidic metabolites were lost. Therefore, we attempted to develop a procedure that would give sufficient clean-up of the sample without losing the acidic metabolites. The use of perchloric acid in the homogenization of brain tissue was considered favourable as the proteins are precipitated, which, after centrifugation, gives a supernatant almost free from proteins. We hoped that it would be possible to inject the supernatant directly onto the chromatographic column without clogging the column top. Subsequent experiments showed that direct injection of the supernatant onto the column was possible and that it was associated with several advantages. In addition to maintaining the acidic metabolites in the injection solution and thus permitting the simultaneous determination of dopamine and its metabolites, the procedure was very fast, giving high recoveries, and thereby reducing the risks of low accuracy and low reproducibility. Our experiments showed that the lifetime of the column was reduced, because the column top slowly deteriorated, but that this process was sufficiently slow to permit the injection of a considerable number of samples. Depending on the injection volume (10–75 μl) and brain region assayed, the column performance was satisfactory for on average 200–600 injections.

Chromatography

Ion-pair chromatography was introduced by Schill *et al.* [5] and later applied to reversed-phase HPLC [6,7]. Increasing the retention of an ionized compound by adding a lipophilic ion of the opposite charge to the mobile phase has been found to be very useful for the regulation of the selectivity between ionized analytes and endogenous compounds. Ion-pair systems were early used for the separation of biogenic amines [8]. In our method, the addition of hexyl sulphate was used to separate the amines, dopamine and epinine, from early-eluting endogenous peaks and to place the amine peaks between the peaks of the acids 3,4-dihydroxyphenylacetic acid and homovanillic acid. It was further shown how this chromatographic system could be used for the separation of several other related compounds, demonstrating a high potential regarding flexibility.

An important difference from earlier methods was the use of a relatively short and efficient column. At this time, the most frequently used columns were μBondapak (300 mm × 4 mm I.D.) with 10-μm particles. Our smaller column (100 mm × 3 mm I.D.) filled with 5-μm particles gave a higher efficiency, resulting in five times less dilution and a corresponding improvement in the detection limits.

Detection

In 1973, a liquid chromatograph with a simple, small-volume electrochemical cell as detector was described [9] and potential applications to the assay of biogenic amines were discussed. This electrochemical cell, which, in spite of its simplicity, was extremely sensitive, became the predecessor of the electrochemical detector, later marketed by Bioanalytical Systems (BAS). Our detector, probably one of the first in Europe, was imported directly from the manufacturer in 1977. The instrument was preceded by a bad reputation and was said to cause endless problems and to need all kinds of "hocus-pocus" to function properly. Typical was the remark of an American visitor at our laboratory: "You will never make it work".

Considering the high sensitivity of the detector and the unprotected position of the electrochemical cell, an early question at issue was the influence of external electrical fields and whether it was necessary to protect the cell from electrical disturbances by placing it in a Faraday cage. At first, it was very difficult to make the detector

function at all. As there was no manual available, trial and error became more or less our investigative method and it took us some months to obtain the first acceptable chromatograms. We then found that the use of a Faraday cage was not necessary. However, the status of the working electrode was crucial. This electrode was made by filling a well in the cell block with carbon paste, followed by extremely careful polishing. This procedure demanded skill, experience and patience because usually it had to be repeated several times before the electrode was usable.

Use of the method and its importance as a tool for pharmacologists

Ever since the direct injection method was first developed in 1978, it has been used continuously in our laboratories and has been an important tool in the search for and study of new antipsychotic and antidepressant compounds.

One of the first compounds tested with the method was remoxipride, a substituted benzamide [10–12]. Various early behavioural tests indicated that remoxipride would have antipsychotic properties with little propensity for giving extrapyramidal side-effects, and this compound was consequently considered as a potential drug against schizophrenia. According to the dopamine hypothesis, schizophrenia is due to dopaminergic hyperactivity in limbic parts of the brain and antipsychotic agents act by blocking dopaminergic receptors. However, when remoxipride was tested in *in vitro* binding studies, it showed only a weak affinity for dopamine receptors. In contrast using our new method to measure the concentrations of dopamine and its main metabolites in brain tissue of rats treated with remoxipride, we found large increases of dopamine turnover. Such studies indirectly demonstrated that remoxipride causes dopamine receptor blockade.

Future directions

Today, more than ten years later, we still use the same method with only minor modifications, such as replacing our previous laboratory-made columns with factory-packed columns. However, the demands made on the method have gradually increased. Previously, most of the samples derived from brain regions with fairly high levels of biogenic monoamines and monoamine metabolites whereas today the bulk of the samples to be investigated are perfusates from microdialysis studies in which the monoamine levels are low. For example, the dopamine concentrations in the dialysate samples are low also when the perfusion takes place in a dopamine-rich region and hence the demands on sensitivity are even further increased when microdialysis is performed in a region with low dopamine neurone density. Often, the small-volume dialysate samples also have to be split to permit analyses for additional compounds, such as GABA or glutamate. An example of a separation obtained from a dialysate sample is given in Fig. 2.

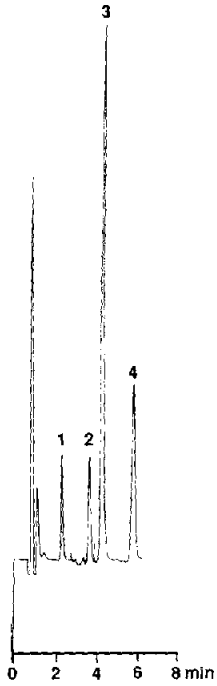

Fig. 2. Chromatogram of a dialysate sample taken from a rat striatum. Column, Supelcosil (7.5 cm × 4.6 mm I.D.) 3-μm C_{18} maintained at 30°C by an LC-22A temperature controller; mobile phase, citrate buffer (pH 4.5) containing sodium hexyl sulphate (2.0 m*M*), EDTA (1.0 m*M*) and methanol (11%); detector, BAS LC-4C set at +0.75 V. Peaks: 1 = 3,4-dihydroxyphenylacetic acid (53.6 pmol); 2 = dopamine (0.17 pmol); 3 = 5-hydroxyindoleacetic acid (13.2 pmol); 4 = homovanillic acid (31.6 pmol). The integrator attenuation was 51.2 nAFs for peak 1, 0.8 nAFs for peak 2 and 6.4 nA full scale for peaks 3 and 4.

Naturally, for the analysis of microdialysis samples maximum sensitivity is a necessity and all precautions have to be taken to obtain optimum conditions. One advantage is that the dialysate samples are fairly clean and therefore precolumns can be excluded, eliminating one possible source of band broadening. However, more important measures may be to use columns with smaller internal diameter. Packed capillary columns and capillary electrophoresis look very promising but there still seem to be problems associated with their routine use. Another, admittedly small but nevertheless important, step forward is the use of columns of 1–2 mm I.D. and packed with 3-μm particles, requiring smaller injection volumes and giving smaller peak volumes than "standard" 4.6 mm I.D. columns. As they can usually be used with standard HPLC equipment, these columns can be utilized directly in most laboratories without the need to purchase additional expensive equipment.

In many laboratories, including ours, on-line *in vivo* microdialysis studies are undertaken. In these studies, perfusion tubes from the treated animal lead directly to the injection valve of the HPLC system and analyses for transmitter and transmitter metabolites are performed continuously. Also in this system high sensitivity and small injection volumes may be required, but an additional demand is reliable automation.

Still in the future for many of us is the vision of determining transmitter and transmitter metabolite concentrations in a single neuronal cell. With a specially designed micropipette and capillary electrophoresis combined with electrochemical detection, this vision has become a reality for Ewing *et al.* [13]. However, the technique is not simple and requires much skill, and at present very few research groups can undertake such studies. Simplification of the methodological procedures involved to permit more laboratories to perform studies in this field is therefore clearly of importance.

CONCLUSIONS

Previously, little help or hope could be given to patients with diseases originating in the brain. The complexity of the human brain seemed to be an insurmountable obstacle to finding medicinal remedies. Therefore, the discovery of the first efficacious therapeutic agents against schizophrenia and depression in the 1950's and their evident effects on dopaminergic, serotonergic and/or noradrenergic neuron systems suddenly changed the generally prevailing pessimism and frustration into a wave of great optimism and belief in the development of new and better drugs. This also resulted in intense research on transmitters and their metabolism coupled with a constantly growing demand for simple and fast analytical methods. The contribution of bioanalytical research to this field is the development of improved analytical methods suitable for preclinical studies and fulfilling such demands as high sensitivity, high throughput and robustness. It is also closely involved in the development of suitable analytical methods for transmitters/modulators where functions in the brain are not yet clear. The importance of this bioanalytical research can hardly be overestimated.

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