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Note

Automated pre-column derivatization with *o*-phthalaldehyde for the determination of neurotransmitter amino acids using reversed-phase liquid chromatography

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The determination of amino acids by high-performance liquid chromatography (HPLC) in combination with pre-column *o*-phthalaldehyde (OPA) derivatization and fluorescence detection [1–3] has gained wide popularity because of its sensitivity, speed and comparative simplicity. Unfortunately, the instability of OPA–amino acid derivatives [4, 5] necessitates precise timing of the reaction and injection sequence and such procedures benefit from automation. Whilst special commercial autosamplers capable of performing this task are now available [6–8], their cost may be prohibitive in many circumstances. Also, although the modification of standard autosamplers to provide pre-column derivatization [9–13] and their application to the routine analysis of complex biological samples [11–13] has been described by other authors these have generally been rather complex or else the reports have lacked detail. The aim of our work was to develop an inexpensive automated HPLC procedure (employing OPA derivatization) by the simple modification of a conventional autosampler that might be utilized by laboratories already in possession of this type of instrument. The procedure and its successful application to the routine measurement of neurotransmitter amino acids in brain extracts and perfusates is described.

EXPERIMENTAL

Materials

HPLC-grade methanol was obtained from May and Baker (Manchester, U.K.). Tetrahydrofuran and 2-mercaptoethanol (MCE) were from Fluka (Fluorochem,

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Glossop, U.K.). Water used for the preparation of buffers and dilution of standards was purified by passage through a deionizing column, an activated carbon column and a sub-micron membrane filter (Elgastat Spectrum SC-1, SC-6 and SC-20 cartridges, respectively; Elga, High Wycombe, U.K.). Crystalline amino acid standards were purchased from Sigma (Poole, U.K.). Other reagents were obtained from BDH (Poole, U.K.) and were of analytical grade except OPA which was Sepramar grade.

Apparatus and derivatization procedure

A Varian LC 5000 liquid chromatograph (Varian Assoc., Walton-on-Thames, U.K.) was used. Separations were performed at 32°C on a column (15 cm×4.5 mm I.D.) packed with Hypersil ODS of 3 μm particle size (Jones Chromatography, Llanbradach, U.K.). The analytical column was protected by a low-volume guard column (2 cm×2 mm I.D.) incorporating 0.5-μm stainless-steel frits and filled with Lichoprep RP-18 of 25–40 μm particle size (Upchurch Uptight; Anachem, Luton, U.K.). The solvents were withdrawn from their reservoirs through 10-μm particulate filters and degassed on-line by an Erma ERC-3510 degasser (HPLC Technology, Macclesfield, U.K.). Fluorescence of the column eluate was continuously monitored using a Varian Fluorichrom filter fluorescence detector (excitation 355 nm; emission 450 nm). Chromatographic data were recorded and processed by a Hewlett-Packard 3390A computing integrator.

Automated sample derivatization and injection was achieved using a modified Magnus M220 autosampler (Magnus Scientific, Aylesbury, U.K.). The sample probe was replaced with a short length of PTFE tubing (0.3 mm I.D.) connected, via a miniature solenoid valve (No. 1200218H, Lee Products, Chalfont St. Peter, U.K.) and a length of stainless-steel capillary tubing (0.18 mm I.D.), to one arm of a low dead-volume T-piece (SSI; Anachem). The opposite arm of the T-piece was connected to a 20-ml vial of OPA–2-MCE derivatizing reagent. The third arm was attached to the inlet port of a pneumatically operated Rheodyne 7010 injection valve fitted with a 50-μl sample loop (see Fig. 1).

A Gilson Minipuls-2 peristaltic pump (Anachem) was used to draw sample and reagent via the T-piece into the sample loop. The speed and duration of pumping were carefully adjusted to ensure (a) adequate mixing of the two fluids, (b) slight over-fill of the loop, but (c) use of minimal sample volume. The ratio of reagent to sample is determined by the relative bores of the connecting tubes, but to achieve the desired sensitivity yet minimize sample consumption, we used equal volumes of reagent and sample. To prevent flow between the sample probe and the reagent reservoir the solenoid valve was only open for the period of the pump activation. A reaction time of exactly 1 min was allowed before the contents of the loop were switched into the solvent stream.

HPLC solvents

Gradients were prepared by mixing two solvents, A and B, modified from those of Jones et al. [3]. Solvent A was 20 m*M* sodium acetate–methanol–tetra-

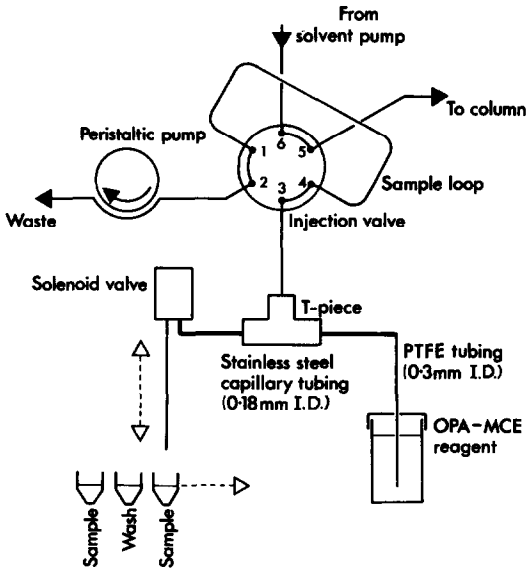

Fig. 1. Diagramatic representation of the automated sample derivatization system. The sequence of operation is as follows. (a) With the injection valve in the 'LOAD' position, sample and reagent are drawn through the sample loop by the peristaltic pump; valve pathway 3-4-1-2 (as shown). (b) Following the reaction period, the valve is pneumatically switched to the 'INJECT' position and the derivatized sample enters the solvent flow to the column; valve pathway 6-1-4-5. (c) After a short delay the valve is returned to the 'LOAD' position in readiness for the next sample while the solvent is continually pumped to the column.

hydrofuran (80:19:1). Solvent B was 20 m*M* sodium acetate–methanol (20:80). Both solvents were filtered through 0.1-μm Nylon 66 membranes (Pall Ultipor N_{66}; Gallenkamp, Loughborough, U.K.) prior to use.

OPA-2-MCE

The OPA–2-MCE reagent was prepared by adding 200 μl of 2-MCE to 19.8 ml of buffered OPA stock solution. To prepare this stock solution 1 g OPA was dissolved in 10 ml methanol before 88 ml sodium borate buffer (24.732 g boric acid dissolved in 1 l water and adjusted to pH 10.4 with 5 *M* sodium hydroxide) were added and the mixture was shaken. Standard solutions containing mixtures of amino acids (1 pmol/μl) were prepared from crystalline amino acids. For analyses based on internal standard calibration a working standard solution of homoserine (Hse; 5 pmol/μl) was used.

RESULTS AND DISCUSSION

Using a simple three-step gradient elution method in combination with variation of the mobile phase flow-rate, it was possible to separate the following amino acids; aspartate (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glutamine (Gln), histidine (His), homoserine (Hse), glycine (Gly), threonine (Thr), arginine (Arg), taurine (Tau), alanine (Ala), γ-aminobutyric acid (GABA) and tyrosine (Tyr). A typical separation of the derivatives of these

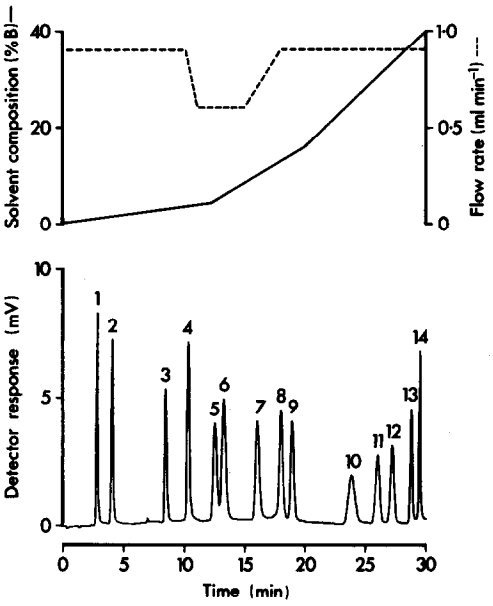

Fig. 2. Chromatogram of a standard (1 pmol/μl) solution of the following amino acids; aspartate (1), glutamate (2), asparagine (3), serine (4), glutamine (5), histidine (6), homoserine (7), glycine (8), threonine (9), arginine (10), taurine (11), alanine (12), γ-aminobutyric acid (13), tyrosine (14). Chromatographic conditions (mobile phase composition and flow-rate) are shown above the trace.

amino acids is shown in Fig. 2 alongside the chromatographic conditions employed.

Reproducible separation was found to depend on several factors, the most important being the need for a period of column reconditioning between injections. In practice, the analysis programme was followed by loop-wash and column-reconditioning steps and samples were injected at 45-min intervals. The loop-wash (deionized water) was included to eliminate sample carry-over between injections. The within-run precision of the automated procedure was evaluated following sixteen consecutive injections of a fourteen-component amino acid standard (1 pmol/μl) using the chromatographic conditions presented in Fig. 2. The coefficient of variation of the retention times of the majority of the amino acids was less than 1% (mean 0.74%), the largest deviation being for Arg ($\pm$10.14 s) and the smallest for Asp ($\pm$1.44 s). The mean variation of the measured peak areas was 2.2%. Hse and Gly showed the largest variation (3.6%) and Asn and Gln the lowest (1.2%).

Normalization of the peak areas to that of the internal standard (Hse) did not reduce the degree of variation; the mean variation of the peak areas for all amino acids (minus Hse) was 3.72% with, and 2.06% without normalization. Although the need for an internal standard has been denied by several authors [10–13], its use has been recommended by others [2, 3, 14]. It is noteworthy that in the former instances automated derivatization was employed whereas in the latter, derivatization was carried out manually. These observations, together with our own, suggest that when automated procedures are used the errors of derivatiza-

TABLE I

LINEARITY OF RESPONSE

Relationship between peak area and amino acid concentration was examined over the concentration range 0.1–5 pmol/μl. The regression equation had the form $y = mx + c$, where y is the peak area (μV s), m is the slope, x is the amino acid concentration (pmol/μl) and c is the y-intercept.

Amino acid	Regression equation	Coefficient of determination (r^2)
Asp	$y = 322611x + 60186$	0.9990
Glu	$y = 353407x + 27176$	0.9994
Asn	$y = 299720x + 28522$	0.9993
Ser	$y = 422988x + 55884$	0.9991
Gln	$y = 434326x + 43101$	0.9990
His	$y = 560070x + 50470$	0.9992
Hse	$y = 469478x + 48409$	0.9990
Gly	$y = 423692x + 63186$	0.9990
Thr	$y = 341038x + 42644$	0.9991
Arg	$y = 284578x + 13332$	0.9994
Tau	$y = 344091x + 27539$	0.9993
Ala	$y = 326693x + 33969$	0.9990
GABA	$y = 276594x + 17413$	0.9994
Tyr	$y = 289009x + 25550$	0.9991

tion and injection are less than those of chromatography and integration.

Routinely, several standards containing the fourteen amino acids shown in Fig. 2 at a concentration of 1 pmol/μl were injected prior to the analysis of experimental samples which were all spiked with a known amount of Hse. The Hse peak was used merely as a retention time marker and as a guide to the gross efficiency of the derivatization and injection procedure. As peaks were identified by retention time, at least one standard was injected every day so that any between-run variations in retention time became unimportant.

The linearity of response was examined over the concentration range 0.1–5 pmol/μl. Linear regression analysis of the peak area of each amino acid versus concentration gave a coefficient of determination of better than 0.999 in all cases (Table I).

The method has been used to study a variety of biological samples including cerebrospinal fluid (CSF), brain extracts and cerebral perfusates from both in vivo and in vitro preparations. Separation of amino acids was good although certain tissue extracts and CSF samples contained so much Gln that the small adjacent His peak appeared only as a shoulder on the larger Gln peak. Several unknown peaks were present, particularly in tissue extracts, but in all cases the majority of components were recognized. A representative separation is shown in Fig. 3.

After prolonged use, and with judicious filtering of solvents and regular guard column maintenance to ensure an acceptable column lifetime, the procedure described has proved to be a sensitive, rapid and robust yet comparatively inexpensive method of automating the detection of endogenous amino acids. Despite its simplicity, our method of pre-column sample preparation compares well with

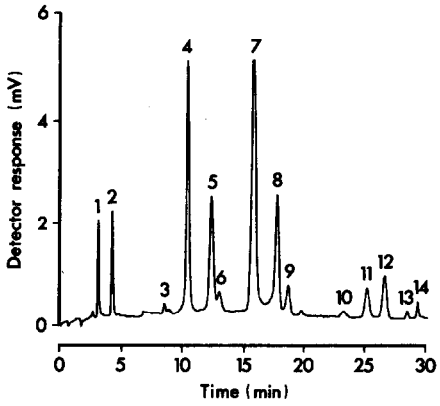

Fig. 3. Amino acid content of perfusate obtained with a push-pull cannula from the prepiriform cortex of a rat anaesthetized with urethane; chromatographic conditions and peaks as in Fig. 2.

more sophisticated approaches and without excessive alteration could prove suitable for other derivatization chemistries.

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