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Note

Problems in the analysis of low levels of amino acids in physiological fluids and tissues using *o*-phthalaldehyde derivatization and reversed-phase high-performance liquid chromatography with electrochemical detection

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The discovery of the general reaction between *o*-phthalaldehyde (OPA) and amino acids in the presence of a reducing agent, generally mercaptoethanol (MCE), to give a highly fluorescent product [1] paved the way to routine analysis of amino acids by high-performance liquid chromatography (HPLC) [2—6]. This method allows the considerable operational advantage of pre-column derivatisation over the post-column derivatisation of many amino acid analysers. The most recent development in such analyses has been the recognition that the isoindoles produced from the amino acids in the OPA reaction are electroactive [7] Several groups have analysed amino acids using electrochemical detection (ED) [8, 9], usually for particular applications [10—12] rather than routine amino acid analysis. The attractions of ED include the possibility of greater sensitivity and selectivity when analysing biological samples. We have expored the analysis of trace amounts of amino acids in biological samples at and below a level of 50 ng ml^{-1} (approx. 300 pmol ml^{-1}) We have shown that, with care, the method is applicable at concentration levels below the detection limit of most fluorescence detectors The limiting factor in the analysis is the amount of background interference that can be tolerated With the development of better instrumentation and the production of purer chemicals than are at present available it will be possible to exploit fully the very great sensitivity and potential of the ED method. With our equipment and using high-quality chemicals we are able to detect a few tens of picograms of derivatised amino acid injected on-column

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EXPERIMENTAL

Materials

OPA (Sigma, Poole, U.K.) was recrystallised from *n*-heptane and stored at −14°C. recrystallisation was repeated every four weeks. Mercaptoethanol (Sigma) was purified by distillation through a 20-cm Dufton column (60°C at 10 mmHg) and taking a heart cut Amino acid standards (Sigma) were dissolved in 2 *M* hydrochloric acid and stored frozen at −14°C Before use they were diluted with methanol—water (30 70) to 10^{-6} g ml^{-1} ^{14}C-Labelled phenylalanine and tyrosine were obtained from The Radiochemical Centre (Amersham, U.K). Chromatography solvents were prepared from HPLC-grade water and methanol (Rathburn, Walkerburn, U.K.) and Aristar-grade (BDH, Poole, U K.) solutes, sodium acetate, anhydrous and glacial acetic acid. AnalaR EDTA (BDH) was used.

Derivatisation

OPA (27 mg) was dissolved in 0.5 ml ethanol and sodium borate buffer (4.5 ml) [7] added. To 1 ml of this solution were added 5 μl MCE to prepare the derivatising reagent. Sample solution (200 μl) was added to an equal volume of reagent, mixed, and allowed to stand for 3 min The mixture was extracted twice with ethyl acetate (1 ml) to remove excess reagents and a 20-μl aliquot injected on-column 5 min after the initial mixing.

High-performance liquid chromatography

HPLC analysis using gradient elution was performed with two 6000A Waters pumps and a Waters 660 solvent programmer (Waters Assoc , Milford, MA, U.S A.) Sample injection was through a Rheodyne 7125 valve (Berkeley, CA, U.S.A) fitted with a 20-μl loop onto a μBondapak C_{18} (10 μm particle size, 25 cm × 5 mm I.D.) column (Waters Assoc.) in an LC-22A temperature controller (BAS, West Lafayette, IN, U.S.A) connected to a Waters 420-E fluorescence detector and an LC-4A electrochemical detector (BAS) with a TL-8A cell*, in series. The cell was set at an oxidation potential of +0.60 V vs Ag/AgCl reference electrode The results were output on a Chessell BD9 recorder and an HP3390A integrator. The mobile phase was constructed from· (A) 50 m*M* sodium acetate in methanol—water (20 80) containing 0.2 m*M* EDTA and adjusted to pH 5.5 with acetic acid; (B) 50 m*M* sodium acetate in methanol—water (70 30) adjusted to pH 5 5 with acetic acid. The high methanol content of B precluded the addition of EDTA. Running conditions were usually a linear gradient (setting 6) from 30% B in A to 100% B over 30 min at a flow-rate of 1 ml min^{-1}

RESULTS AND DISCUSSION

The analysis of amino acids after conversion to isoindoles is rapidly

*This device is extremely sensitive to external fields when operated at high sensitivity and shielding additional to the Faraday cage supplied by the manufacturer is often necessary When PTFE tubing is used for inlet and outlet these should be shielded and the shielding earthed We use an earthed metal sheathing either copper tubing (3 mm O D), though this is relatively inflexible, or the woven shield from co-axial or microphone cable

becoming a standard method It is quick, convenient and relatively easy when large samples are available (> 10 μg). We have shown that the method is still applicable for very low concentrations of amino acids in samples (< 1 pmol) when using ED and that the limits of detection have not yet been reached. Problems associated with the analysis increase with diminishing sample size and are largely due to impurities in reagents and running solvents and the inherent lack of chemical stability in the isoindoles examined in the reaction.

Initial attempts to analyse standard amino acid solutions at concentrations below 1 μg ml^{-1} were frustrated by background interference which masked several of the amino acid peaks Much of this interference was shown to derive from the MCE· freshly supplied samples gave the least interference and "old" samples were so bad as to be unusable Careful distillation in subdued light at low temperature and under vacuum taking a heart cut gave a product which in the assay gave a lower overall background, and from the chromatogram of which certain discrete peaks were missing. The first fraction, the tailings and the residue from the distillation were none of them better than undistilled material The heart cut, when stored in the dark at room temperature, had not noticeably changed over several months.

Another potential source of interference was the water used in the running solvent. Only the most highly polished, or commercial HPLC-grade, water is satisfactory for high-sensitivity analysis If a good grade of water is used in the running solvent there will be little change in the averaged background throughout a gradient run. Further reduction in the background is achieved by using

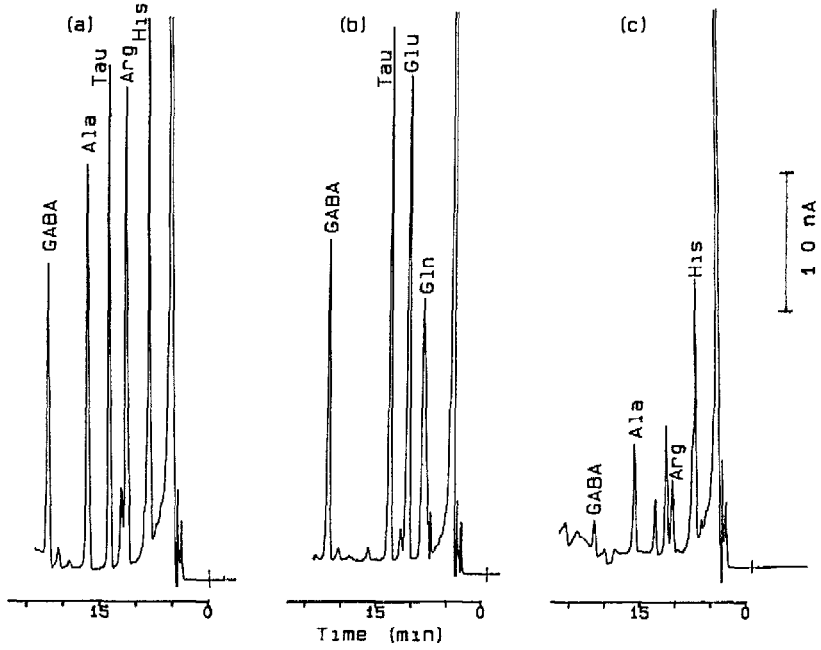

Fig 1 Chromatograms of groups of amino acid standards (a and b) 25 pmol of each on-column, (c) 250 fmol of each on-column The greater sensitivity in this tracing results from a change in the electrode surfaces Column temperature, 25°C, detector sensitivity, 5 nA f s d

the highest grade of solutes available Analytical-grade solutes may not be sufficiently pure to be used in high-sensitivity ED assay With these precautions 25 pmol of an amino acid injected on-column is easily quantitated and 1% of this is detectable (Fig 1) for selected amino acids sub-picomole levels may be quantitated. At the 25 pmol level intra-assay variability for replicate analyses was within the range 4 6 ± 2% for each amino acid measured and inter-assay variability was 4.8 ± 3% (n = 14) according to the amino acid measured

The stability of the isoindole formed in the reaction of OPA with an amino acid during chromatography was investigated using radioactive phenylalanine and tyrosine. The radioactive purity of the compounds was first established by thin-layer chromatography and autoradiography to be > 99%. When submitted to the assay, in carrier at a total concentration of ca. 1 μg ml^{-1} (5 nmol ml^{-1}) and the column eluent counted, taking 0 5-ml aliquots, radioactivity above background was found in all fractions after the void volume While the peak shape was good for each amino acid it contained less than half of the total radioactivity injected on-column In the case of phenylalanine, which eluted after 16.9 min, approx. 40% of the injected radioactivity was in the phenylalanine peak The short half-life of these compounds has been noted by other authors but this experiment shows that breakdown of the amino acid derivative on-column produces a roughly constant high background and that the analysis time must be short for maximum sensitivity Therefore the full potential of this method of amino acid analysis may only be realised with micro-bore columns and the associated technology

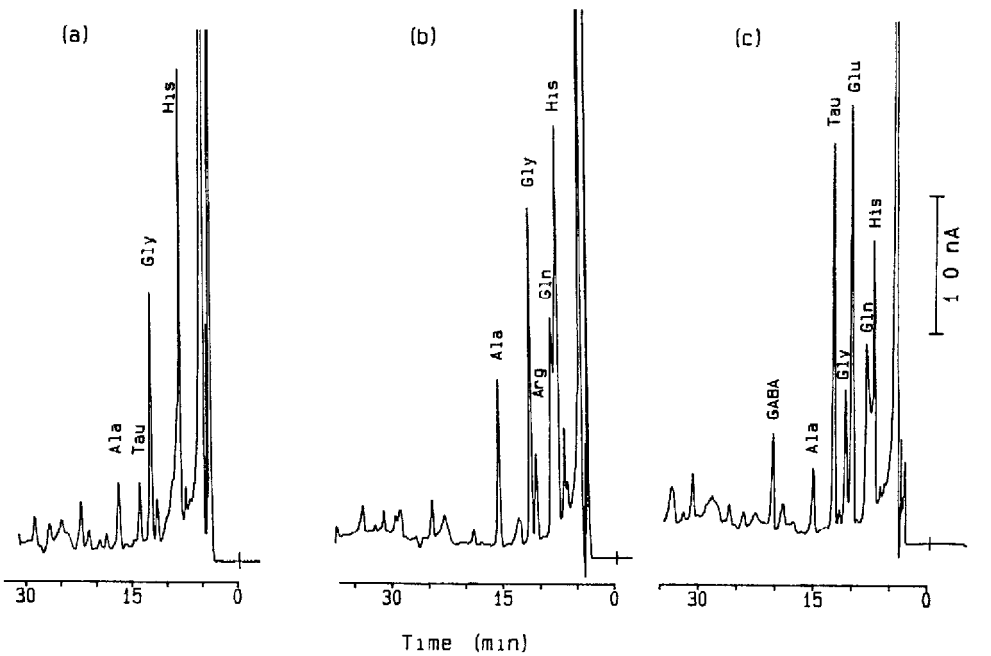

Fig 2 Chromatograms of the amino acids in biological samples (a) urine, dilution 1 1000, equivalent to 4 nl urine injected on-column, (b) plasma, dilution 1 1, equivalent to 2 μl plasma injected on-column, (c) mouse brain homogenate, dilution 1 4000, based on tissue, equivalent to 1 ng brain injected on-column Conditions as in Fig 1

The assay has been used to study a variety of biological samples including urine (Fig 2a), plasma (Fig 2b) and brain (Fig. 2c) The aim at this stage is to show the sensitivity of the detection method rather than to advance the chromatography, which is already well described A limiting factor in the handling of biological samples is the quantity of material that may be successfully manipulated. There are many occasions when analysis of a small sample is necessary. Human urine is generally presented to a laboratory in embarrassingly large quantities but the amount that may be taken from a foetus, for example, or collected from a small animal could be less than 100 μl in volume. In human studies analysis of small (50—100 μl) plasma volumes means that a finger prick rather than a venepuncture is all that is required for sample collection. When pharmacokinetic or metabolic studies are carried out using small animals only small volumes of blood are available with repeated sampling This has the added economic and other advantages that fewer animals are required for any experiment

We have shown that analysis of amino acids in biological samples using OPA derivatisation and ED is possible and satisfactory using samples containing nanogram quantities of amino acids These levels do not represent the limit of detection, rather they are a working range that can be attained, at the present time, if attention is paid to the details of the method

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