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ELECTROCHEMICAL ACTIVITY OF *o*-PHTHALALDEHYDE—MERCAPTOETHANOL DERIVATIVES OF AMINO ACIDS

APPLICATION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS IN PLASMA AND OTHER BIOLOGICAL MATERIALS

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SUMMARY

o-Phthalaldehyde—mercaptoethanol (OPA) reagent is used to increase the sensitivity of post-column detection of amino acids after chromatographic separation. OPA-amino acids themselves chromatograph well on reversed-phase high-performance liquid chromatographic (HPLC) columns and can be determined by fluorimetric detection, enabling OPA to be used as a pre-column derivatisation reagent. In this paper electrochemical detection (in the 0.4—0.7 V range) of OPA-amino acids following reversed-phase HPLC is described. Using fluorimetric and electrochemical detection in series, confirmation of the identities of the amino acids can be obtained in a single chromatographic run. Particular amino acids (e.g. basic amino acids) are active at the lower potentials, so that they can be selectively detected if required. Other amino acids and peptides whose OPA derivatives have little or no fluorescent activity are electroactive, permitting their detection by HPLC of their OPA derivatives.

Application of this methodology to divers biological samples is illustrated. OPA derivatisation of amines is an example of a reaction in which the product is electroactive at a lower potential than the reactants. This type of reaction is likely to be particularly useful in extending the applications of electrochemical detection in HPLC.

INTRODUCTION

The sensitivity of traditional ion-exchange procedures for amino acid determination has been enhanced by the use of fluorogenic derivatisation reagents. Of these, *o*-phthalaldehyde—mercaptoethanol (OPA) reagent is notable because of its cheapness and its convenience due to its lack of intrinsic fluorescence,

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and its stability in water [1—3]. Polar amino acids are poorly retained on reversed-phase high-performance liquid chromatographic (HPLC) columns, but Lindroth and Mopper [4] showed that OPA derivatives of amino acids are well retained and resolved on reversed-phase columns. These authors and subsequently others [5, 6] described the determination of amino acids in biological materials, following pre-column derivatization with OPA, using reversed-phase HPLC with fluorescence detection.

Electrochemical detection (ED) provides a sensitive and specific method for compounds possessing intrinsic electrochemical activity. We [7, 8] and others [9, 10] have previously discussed the advantages of series fluorescence and electrochemical detection. Most amino acids are not intrinsically electrochemically active within the currently useful potential range, with the exception of tryptophan, tyrosine and cysteine [11]. The structure of OPA-amino acids derivatives contains an isoindole grouping [4]. In view of the electrochemical activity of indoles (such as tryptophan) we set out to determine whether OPA derivatives of the amino acids could be detected electrochemically. In this paper we show that they can be, and demonstrate the HPLC analysis of amino acids in various materials, using fluorimetric and electrochemical detection in series.

MATERIALS AND METHODS

Chromatography

A 15 cm × 4.6 cm column, packed with Hypersil ODS 5 μm, a guard column type A (both from Chrompack, London, U.K.) and phosphate buffers with methanol as organic modifier were used. For isocratic elution an Altex 110A pump was used with 0.1 *M* sodium phosphate buffer (pH 7.0)—AnalaR grade methanol (50:50) as eluent. For gradient elution two Kontron 410 pumps controlled by a Kontron 200 programmer were used. The eluent was a mixture of 0.05 *M* sodium phosphate buffer (pH 5.5)—AnalaR grade methanol in the proportions 80:20 for solvent A and 20:80 for solvent B. Samples were introduced using a 7125 Rheodyne injection valve with a 20-μl loop. Fluorescence (Kratos Fluoromat 950, excitation: 365 nm band pass filter, emission: 470 nm cut-off filter) and electrochemical (Bioanalytical Systems TL5 and LC-4) detectors were used in series. Chromatographic equipment was supplied by Anachem. (Luton, U.K.) (Altex, Bioanalytical Systems), Konton (St Albans, U.K.) and Kratos-Schoeffel (Manchester, U.K.).

Analytical procedure

OPA reagent was made up in the proportions described by Lindroth and Mopper [4] by dissolving 27 mg of *o*-phthalaldehyde in 500 μl absolute ethanol, and adding 5 ml of 0.1 *M* sodium tetraborate (in place of 0.4 *M* boric acid brought to pH 9.5 with sodium hydroxide [4]) followed by 20 μl of mercaptoethanol. The reagent was kept overnight before use, and 10 μl mercaptoethanol added if required (each 1—2 weeks) to maintain maximal yield.

Plasma (50 μl) was deproteinised with 4 volumes of AnalaR grade methanol by thorough mixing, standing for 10 min at 4°C and centrifugation for 5 min

at 8000 *g*. Water (50 μl), or mixtures of appropriate standard amino acid solutions (typically 100 nmol/ml final strength) were carried through the same procedure. One volume of supernatant was reacted with 4 volumes of OPA reagent at room temperature. At a timed 2-min interval after mixing the reactants, a 20-μl aliquot was injected into the HPLC system. Other biological materials were also studied at appropriate dilutions and with suitable standards. Cerebral spinal fluid (CSF) was diluted up to 5 times with methanol, and reacted as above. Brain was homogenised in 10 volumes of methanol and the supernatant reacted as above. However, further dilution of up to 10-fold was needed for the more abundant amino acids. Brain perfusates, in contrast, were reacted directly with an equal volume of OPA reagent. Gastric juice required up to a 5-fold dilution in addition to that of the standard procedure for plasma.

Standard amino acids, *o*-phthalaldehyde, mercaptoethanol, tryptophyl dipeptides and tryp-met-asp-pheNH_2 were obtained from Sigma (Poole, U.K.) and 3-hydroxykynurenine from Koch Light (Slough, U.K.). Bulk reagents were obtained from BDH (Poole, U.K.).

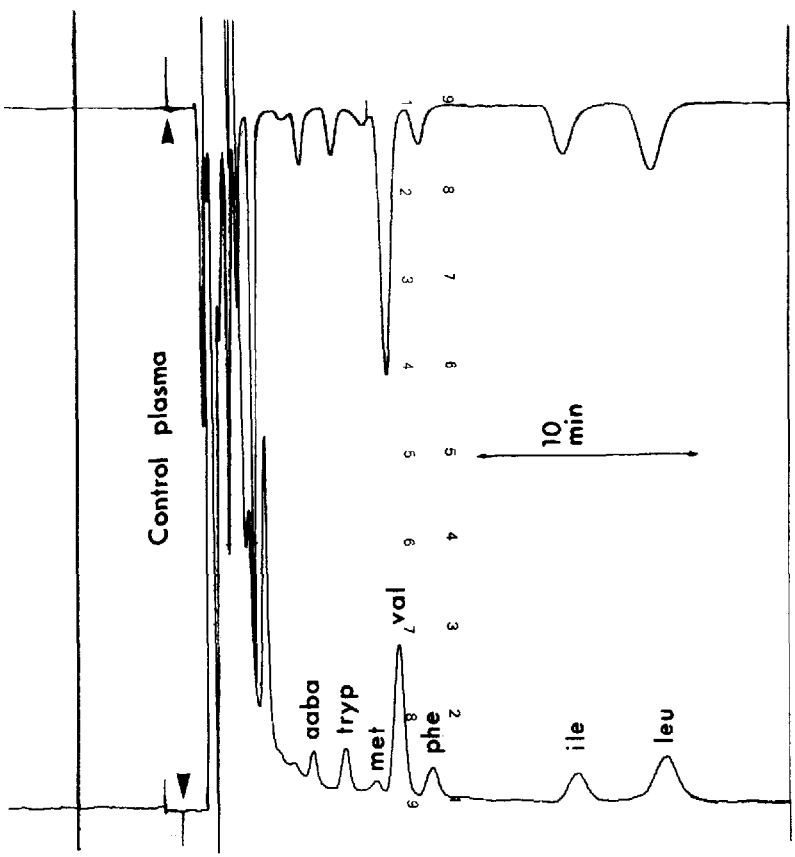

Fig. 1. Isocratic elution of large neutral amino acids in human plasma. Injection of 20 μl: equivalent to 1 μl plasma. Lower: ED trace at 1.0 V, f.s.d. = 100 nA; upper: fluorimetric trace (fluoromonitor 50 mV, × 10 setting). (Pen offset 24 sec in all chromatograms.)

RESULTS AND DISCUSSION

Using the isocratic conditions described, good resolution of a group of slower running (i.e. more hydrophobic) OPA-amino acid derivatives was achieved in about 20 min. Those detected in plasma were α-amino butyric acid (aaba), tryptophan (tryp), methionine (met), valine (val), phenylalanine (phe), isoleucine (ile) and leucine (leu) (Fig. 1). Recoveries of standards added to plasma were 95—100%. Each peak on the fluorescence detector was accompanied by an electrochemical peak with the working electrode set at 1.0 V (since the half-wave potential was expected to be in the range of the indole group). This group of amino acids in plasma is of interest, since it includes all but one (tyrosine, tyr) of a group of large neutral amino acids which compete with one another for uptake by the brain, affecting the rate of amino neurotransmitter synthesis [12].

For resolution of the full range of amino acids we utilised gradient elution (Fig. 2). Due to the higher methanol concentrations reached the molarity of the phosphate buffer was reduced by half. We were particularly concerned to resolve histidine (his) from glutamine (glu) for a particular application, and to resolve γ-aminobutyric acid (gaba) from alanine (ala), for use in brain studies. This was achieved by reducing the pH to 5.5 and utilising a gradient of 0 to 10% B over 10 min, then 10 to 85% B over 30 min, followed by 85 to 0% B in 5 min and 10 min re-equilibration. Resolution of asparagine (asp) from glutamate (glu) and tryptophan from methionine are incomplete. Variation of

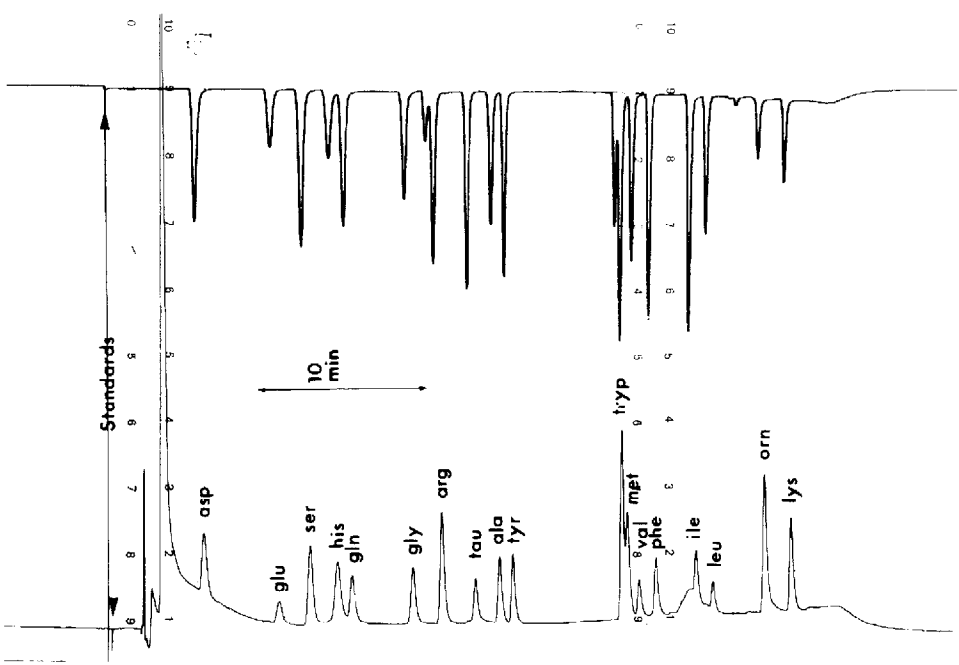

Fig. 2. Gradient elution of amino acid standard mixture 400 pmol each on column. Lower: ED trace at 0.5 V, f.s.d. = 50 nA; first peak is due to reagent (see text); upper: fluorimetric trace (Fluoromat 10 mV, × 1 setting).

the buffer species and its molarity, and of the pH and of the gradient profile permits optimisation for particular applications.

It is often stated that gradient elution is incompatible with ED. We have previously reported [13] that this is not the case for step gradient elution, provided that precautions are taken to minimise metal ion contamination of the eluent. The same holds for continuous gradient elution, the principal modification required being the substitution of sintered glass for metal frits on the solvent inlet lines.

The chromatogram of standard amino acids in Fig. 2 shows that with gradient elution also each fluorescence peak is accompanied by an electrochemical peak. Variation of the operating potential of the electrochemical detector revealed that the isoindolic OPA derivatives are active at quite low operating potentials (in this case 0.5 V) in contrast to the indoles and phenols which require a potential of 0.8—1.0 V. At this lower potential, baseline disturbance due to the gradient is small, as is the unretained peak. The ED trace from a blank also shows fewer interferences than the fluorimetric detector trace.

Since the fluorescent and electrochemical peak heights do not co-vary across different amino acids, the ratio of fluorescent to electrochemical activity at a

TABLE I

RATIOS OF FLUORESCENT TO ELECTROCHEMICAL ACTIVITY FOR AMINO ACIDS IN STANDARD MIXTURE AND IN HUMAN PLASMA

Amino acid	FL/$EC_{0.5}$ ratio*	
	Standard	Plasma
Isoleucine	4.47	5.43
Leucine	4.00	4.17
Valine	3.54	3.40
Phenylalanine	3.50	3.83
Taurine	3.45	3.88
(α-Aminobutyric)		2.70
Tyrosine	2.51	2.75
(Asparagine)		2.44
Glutamine	2.53	2.24
Glutamate	2.36	2.42
Aspartate	2.00	
(Threonine)		2.00
Methionine	2.00	
Alanine	1.92	1.78
Glycine	1.85	1.81
Serine	1.83	1.85
Arginine	1.45	1.50
Histidine	1.08	1.15
Lysine	0.82	0.80
Tryptophan	0.69	0.67
Ornithine	0.42	0.48

*FL/$EC_{0.5}$ ratio is the ratio of peak height on the fluorimetric detector at 10 mV × 1 to that on the electrochemical detector at 0.5 V, f.s.d. = 50 nA (settings of Figs. 2 and 3).

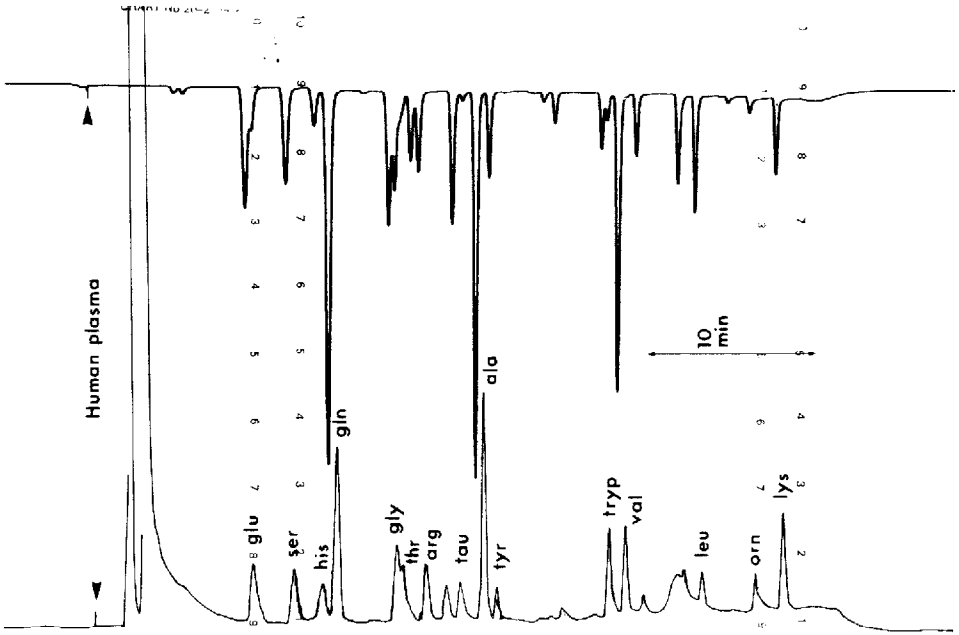

Fig. 3. Gradient elution of amino acids in human plasma. Conditions as Fig. 2.

given potential can be used to characterise the different amino acids. Table I shows the good agreement of the ratio obtained for standard amino acids, and for the amino acids in a human plasma sample (Fig. 3; the relatively poor agreement for isoleucine is probably due to interference on the ED trace).

If the operating potential is varied on successive runs, the electrochemical/fluorescence ratio for each amino acid increases with the potential, as expected. However, different amino acids show different profiles of activity (equivalent to different spectra). Thus at 0.4 V (Fig. 4A; same standard mixture as Fig. 3, ED sensitivity increased 2.5-fold) the basic amino acids retain a greater proportion of their electrochemical activity than the other amino acids. Conversely, differences between the amino acids are reduced at 0.6 V (Fig. 4B). If the operating potential is systematically varied from 0.4 to 0.7 V then profiles such as those shown in Fig. 5 are obtained.

OPA-amino acid derivatives show a less steeply increasing electrochemical activity with working potential (Fig. 5) than do simpler compounds, (e.g. catechols, indoles) presumably due to the interaction of multiple oxidisable groups in each derivative. The curves for the different amino acids fall into groups related to the chemical structure of the amino acid residue. The groups into which the profiles fell (of which space permits only representative examples in Fig. 5) include, in approximately ascending order of electrochemical/fluorescence ratio: 1, large neutral (val, leu, ile, phe) and taurine; 2, acidic (glu, asp); 3, small neutral (gly, ala, ser, thr); 4, basic (arg, his, lys, tryp, orn); 5, atypical: some amino acids show a steeper profile (tyr, his).

These observations imply that the amino acid residues, even those that are not intrinsically electroactive, will affect the electrochemical profile of the S-

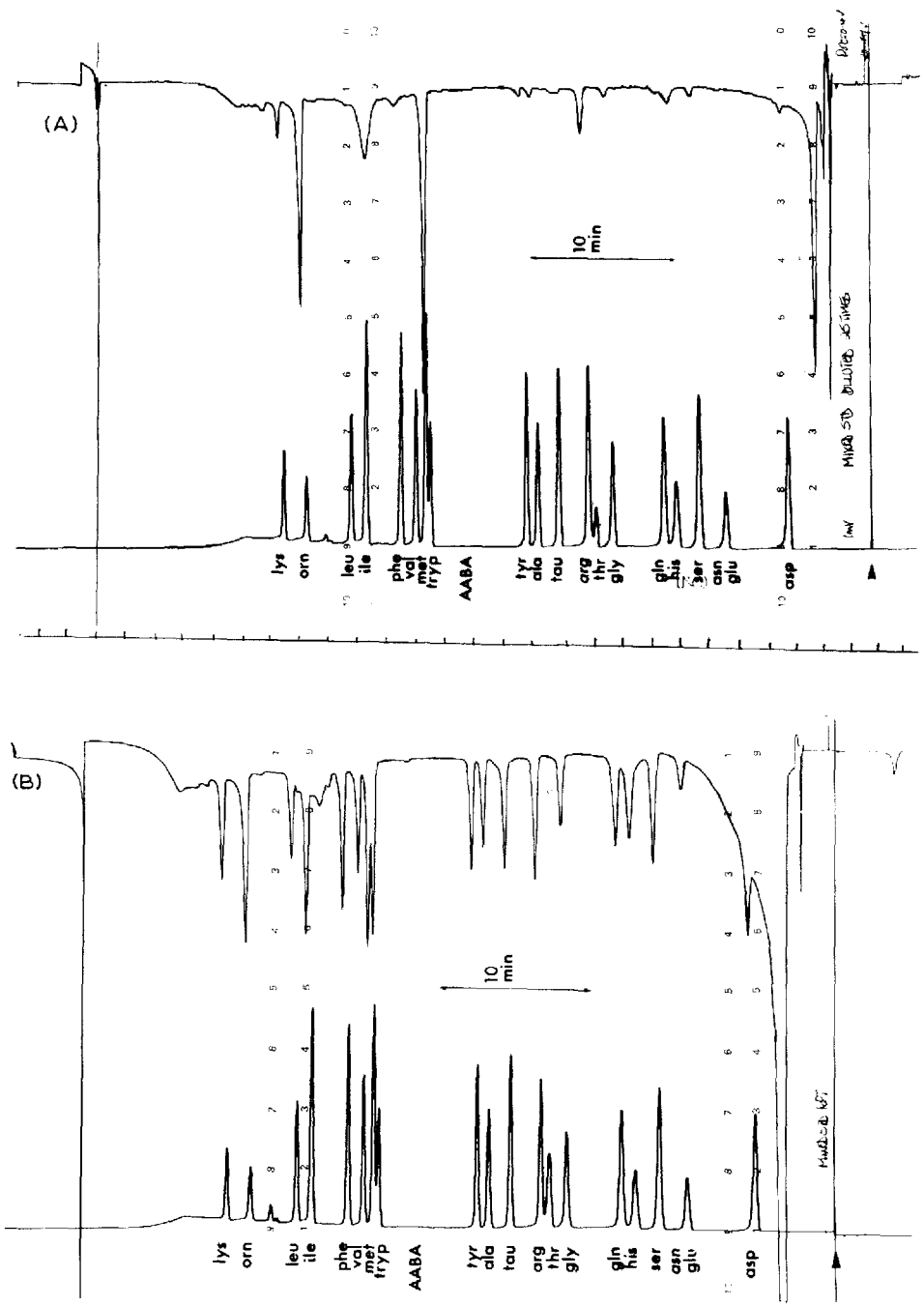

Fig. 4. Variation of electrochemical detector potential. Standard amino acid mixture, and lower (fluorimetric) trace exactly as Fig. 2; upper (ED) trace (A) at 0.4 V, f.s.d. = 20 nA; (B) at 0.6 V, f.s.d. = 50 nA. (N.B.: time zero at right.)

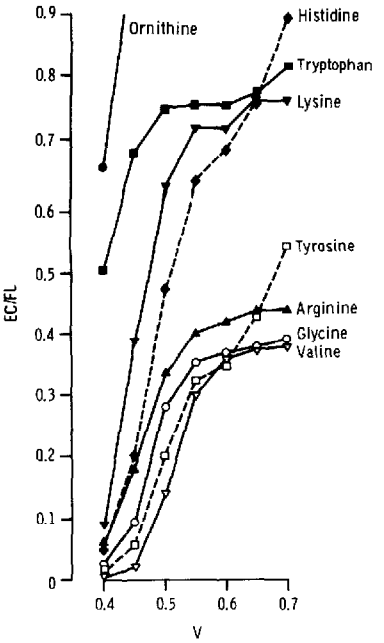

Fig. 5. Variation of electrochemical activity with working electrode potential. Vertical axis: peak height ratio of ED response (corrected to 50 nA f.s.d.) to fluorimetric response (at 10 mV × 1) for equimolar amounts of the amino acids stated.

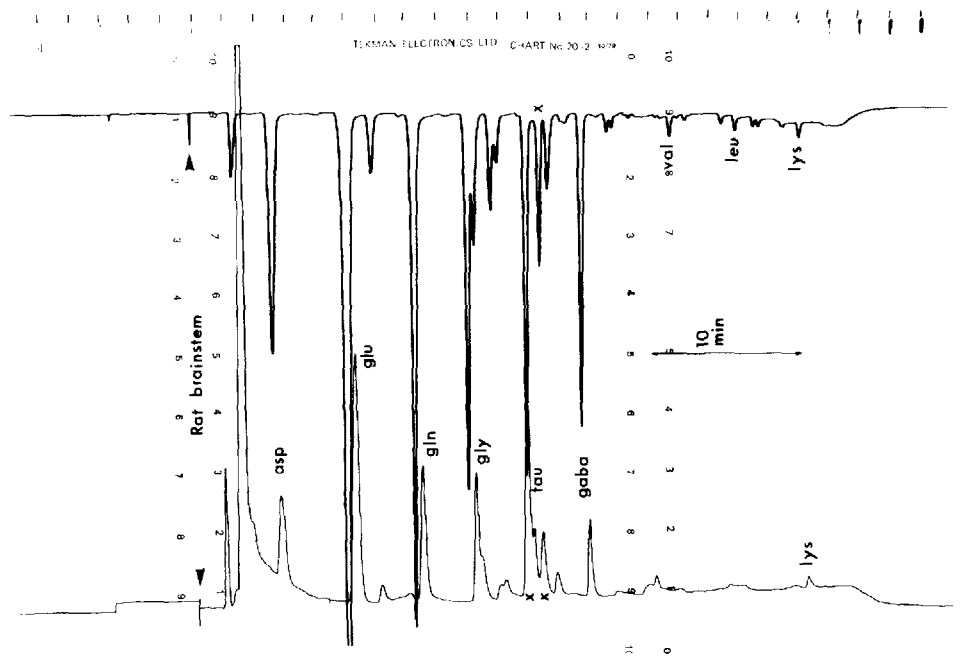

Fig. 6. Amino acids in rat brainstem. Conditions as Fig. 2, but fluorimetric detector, × 0.5 setting.

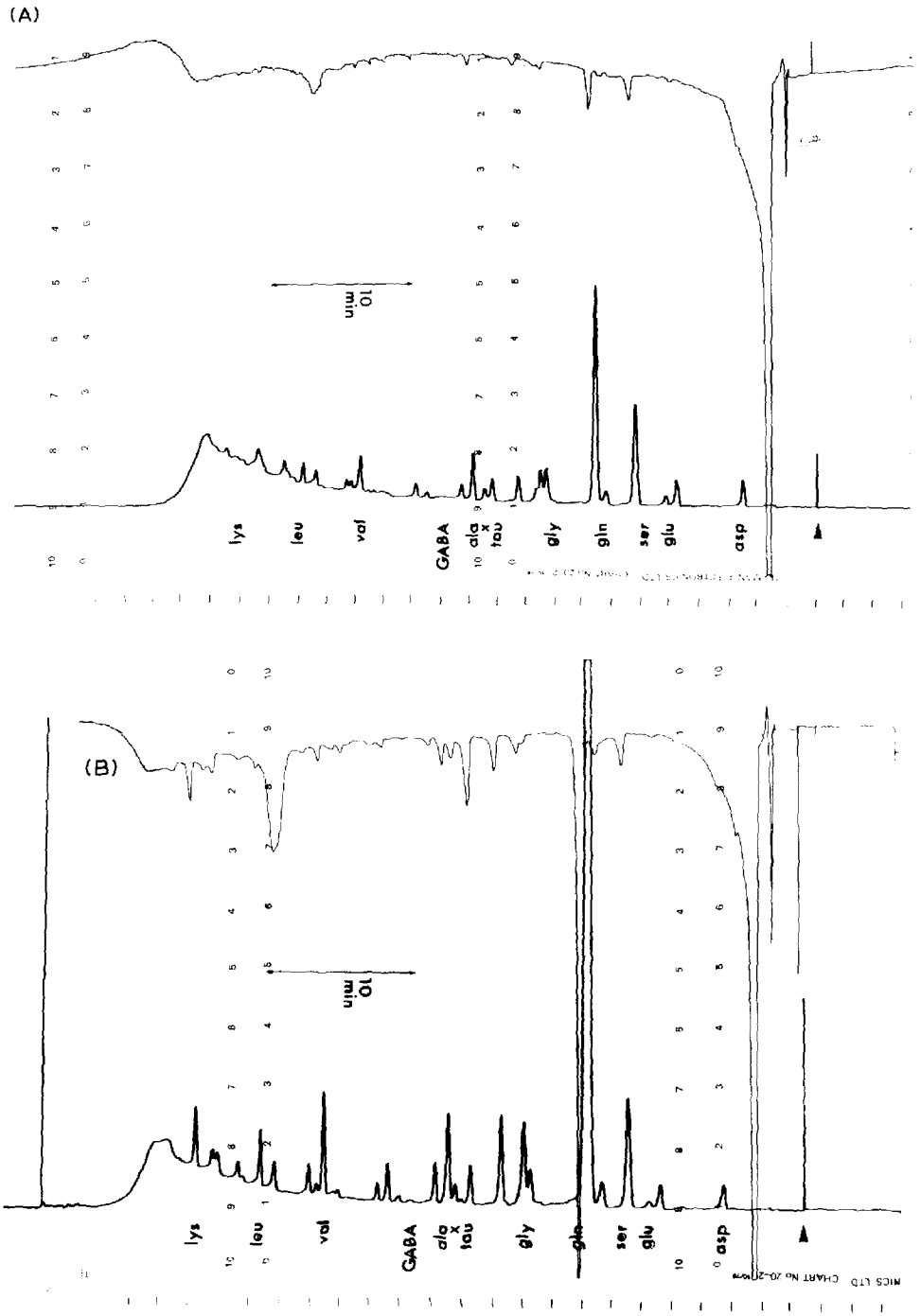

Fig. 7. (A) Amino acids in push—pull perfusate of rat hippocampus. Conditions: upper (ED) trace at 0.5 V, f.s.d. = 20 nA; lower fluorimetric trace 10 mV × 0.2 setting. (B) Amino acids in human lumbar CSF. Conditions: as A but ED trace (upper); f.s.d. = 50 nA.

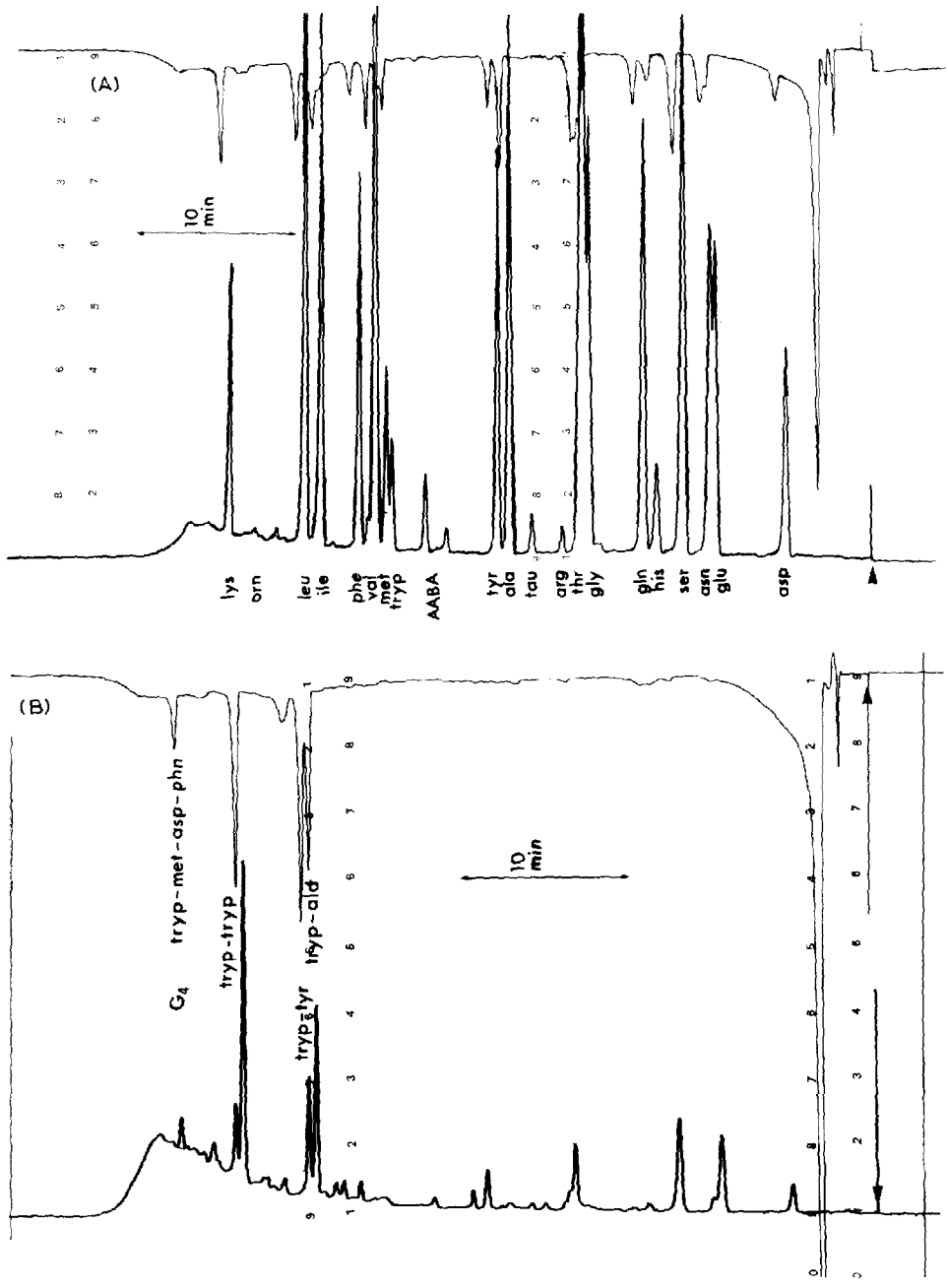

Fig. 8. (A) Amino acids in human gastric juice. Conditions: as Fig. 7B, but fluorimetric trace (lower) at 10 mV × 0.5 setting. (B) Separation of tryptophyl di- and tetra-peptide standards. Conditions as in Fig. 7B, but fluorimetric trace (lower) at 10 mV × 0.1 setting.

substituted isoindole which is common to all the derivatives. From inspection of Fig. 5 it can be anticipated that setting the potential at 0.5 V gives the most even spread of fluorescence to electrochemical ratio across the different amino acids. This indeed was the case in our hands, and is the basis of the selection of the $FI/EC_{0.5}$ ratio (see legend to Table I) in compiling Table I.

Figs. 6—8A demonstrate the application of the method to a variety of biological samples; rat brain (tissue extract), rat brain push—pull perfusate [14], human CSF and human gastric juice. The brain amino acid pool is dominated by aspartate, glutamate, glutamine, glycine, taurine and gaba. Since these are all among the faster running amino acid derivatives, an accelerated gradient could be used for rapid determination of brain amino acids. In this way the method can be modified to suit particular requirements. Electrochemical together with fluorimetric detection of OPA-amino acids offers improved versatility and specificity in the HPLC of the amino acids commonly found in biological materials.

OPA derivatives of a number of amino acids, including cysteine, kynurenine and 3-hydroxykynurenine show little or no fluorescence at the wavelengths usually employed for fluorimetric detection. These amino acids do however form derivatives, since we are able to detect them electrochemically (results not shown). OPA derivatives of the N-terminal amino acid of small peptides are also formed, but show marked fluorescence quenching compared to single amino acids. Fig. 8B shows the separation of standards of tryp-ala, tryp-tyr, tryp-tryp and C-terminal tetrapeptide common to gastrin and CCK, tryp-met-asp-phe-NH_2 (G_4). Variation of the working potential gave the results in Fig. 9, showing that while the fluorescent yield of the OPA derivative falls by some 32-fold in going from tryptophan to G_4, the electrochemical yield falls by 16-fold at 0.4 V and only 3-fold at 0.55—0.60 V. Expressed another way, ED at settings which give approximately equally sensitivity to fluorimetric detection for tryptophan, gives 3 to 8 times more sensitivity than fluorimetric detection for the dipeptides and 8 to 12 times more sensitivity than fluorimetric detection for G_4.

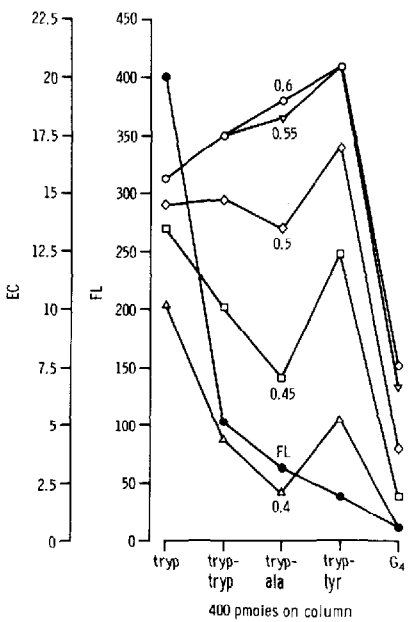

Fig. 9. Electrochemical and fluorescent activity with increasing peptide length. ED: peak height in nA at stated potential; fluorimetric detection: peak height in mm (200 mm = 1 mV).

Thus, in addition to its application to the HPLC determination of the usual amino acids, ED offers considerable promise for the determination of OPA derivatives of other amino acids and small peptides following HPLC separation. In addition to its specific application to the amino acid/peptide field, it is anticipated that the general technique, of which this is an example, of making derivatives that are electrochemically active in a range markedly lower than that of the parent species and of the derivatisation reagents will enable the scope of ED to be considerably enlarged.

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