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Determination of peptide 520 in human plasma using post-column photolysis with electrochemical detection in liquid chromatography

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Abstract: A simple LC method for the determination of peptide 520 in human plasma was developed. Based on micellar chromatography, sodium octyl sulphate (SOS) was added into the mobile phase in order to separate the peptide from human plasma components. The procedure was fast and sensitive for the determination of the peptide in untreated human plasma. The electrochemical (EC) detection limit for peptide 520 in human plasma was 0.5 μg ml^{-1}. Linearity of the calibration plot for peptide 520 in human plasma was 0.999. This approach represents a direct injection technique for the potential detection and analysis of numerous peptides in biofluids, besides just plasma, with absolute quantification.

Keywords: *Reversed-phase HPLC; electrochemical detection; photolytic derivatization; micellar mobile phases; sodium octyl sulphate.*

Introduction

The application of LC with EC detection is very attractive because a number of biological processes involve an electrochemically active species. LC–EC has shown high sensitivity, excellent selectivity and a wide linear dynamic range in the areas of clinical chemistry, pharmaceutical analysis and drug determinations [1–2]. Usually, only those analytes which have inherent electrochemical activity can be directly determined by EC. Aromatic and sulphur-containing amino acids, such as tyrosine, tryptophan, methionine and cysteine; and some small peptides which contain these amino acids, can be measured by EC without prior derivatization [3–5]. On the other hand, other amino acids and peptides or even proteins can be determined indirectly by using derivatization [6–7].

There are some approaches which are used to determine nonelectrochemically active amino acids, peptides and some proteins: pre- or post-column chemical derivatization (PCCD); chemically modified electrodes (CME); electrical polarization (EP) and pre-, or post-column photolysis (PCP). First of all, PCCD is normally used in the absence of a CME. Amino acids and peptides react with any nitroaryl reagent in either solution or solid-phase reactions to generate EC active derivatives, amenable to direct reductive or photolytic, oxidative methods [6–10].

Secondly, the use of CME has extended the applications of LCEC [11]. The difficulty of direct oxidation and reduction of peptides and proteins at conventional electrodes can, at times, be overcome by using CME. Examples are the mercury–gold amalgam, nickel and copper oxide electrodes, and cobalt phthalocyanine electrodes [12–15]. The formation of a complex between the metal and the amino acid leads to the dissolution of a passivating film formed on the metal surface, which results in the observed current. Another example is the use of polymer electrodes which were coated with conducting polymers. Immobilizing reversible redox bioplymers on an electrode surface can provide a route to permit amperometric detection of many species or decrease the high applied potential necessary for some analytes [38]. The detection of amino acids is based on the surface-catalysed oxidation of the amine functionality, which is activated by the formation of metal oxides [11–20]. The disadvantage of this method is a difficulty in

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controlling the kinetics of complexation between metals and amino acids. Another problem is the need for high operator skill, in order to make a good working electrode surface. Furthermore, most of the electrode materials are not commercially available.

The third method is polarization [21–22]. It is based on the application of anodic and cathodic polarization to clean and continuously reactivate the electrode surface by using a cyclic potential sweep. This procedure can solve the rapid loss in performance by direct detection at noble metal electrodes, due to passivation of the electrode surface [21]. However, sometimes CME are still needed to decrease the high applied potential for reduction or oxidation on some working electrodes [22].

The fourth way is to use photolysis [23]. Using irradiation as the derivatization source is practical and easy. In order to improve the EC detection of amino acids and peptides, our laboratory has developed an on-line, post-column, photohydrolytic or photolytic reaction in LC–EC systems [23–28]. By using an improved, knitted open tubular reactor for the photochemical reactions, some amino acids and peptides having no native EC properties can be readily detected after irradiation. Therefore, some peptides or even three-dimensional proteins with aromatic and sulphur-containing amino acid components showed EC responses after photolysis. By using on-line, post-column photolysis as a derivatizing reagent, the application of EC has been extended from small, electrochemically active analytes to larger, three-dimensional structured proteins, which have some electrochemically active residues folded inside [27–28, 37].

The need for highly selective and sensitive methods for the determination of drugs in biological fluids is increasing. The direct injection of biological fluids onto a HPLC column has been widely employed, since micellar mobile phases are able to solubilize proteins and thus prevent their precipitation and column plugging [29–30]. The advantage over traditional hydro-organic mobile phases include the ability to simultaneously chromatograph hydrophilic and hydrophobic compounds, the lower cost and greater safety of micellar mobile phases, as compared with conventional mobile phases containing organic solvents, and the greater solubility of solutes that can be used to control ionic strength, pH and buffering capacity in micellar mobile phases. Another major advantage of using micellar mobile phases is the complete elimination of *all* sample preparation steps. A rapid and sensitive LC analysis, with EC detection, has been developed for the simultaneous determination of some drugs in human plasma, dog plasma and urine, but only used for electrochemically active drugs [31–36]. There are two surfactants which have been used in micellar mobile phases for the determination of drugs in biological fluids: sodium dodecyl sulphate (SDS) and polyoxyethylene dodecanol (BRIJ 35) [29]. Both surfactants are suitable for small drugs in biological fluids. However, when these surfactants are added to mobile phases, significantly decreased chromatographic efficiency for some peptides in biological fluids is seen because of poor mass transfer between the micelle and stationary phase [38–41].

Experimental

Apparatus

The instrumentation was composed of three parts: a conventional LC system with a UV detector, a photolysis unit, and a conventional amperometric EC detector, as described in previous papers [23]. The LC system consisted of a Model 590 solvent delivery pump (Waters Chromatography, Millipore, Milford, MA), a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA) with a 20-μl sample loop, a 150 × 4.6 mm Supelco LC18-DB column and a 2 cm Supelguard LC-18 pre-column (Supelco, Bellefonte, PA), and a Model LC75 spectrophotometric detector (Perkin-Elmer, Norwalk, CT). The post-column, on-line photolysis unit was a Model Beam Boost photochemical reaction unit (Astec, Whippany, NJ), with a low pressure mercury lamp having a main irradiation line at 254 nm. A knitted open tubular (KOT) reactor equipped with a 0.3 mm i.d. × 1.6 mm o.d. (10 m) Teflon tubing (Alltech Associates, Deerfield, IL) was wrapped around the UV light source. The EC detector was a Model LC-4B dual electronic controller with a dual glassy carbon working electrode (GCE), a stainless steel auxiliary electrode, and a Ag–AgCl reference electrode, all obtained from Bioanalytical Systems, Inc. (West Lafayette, IN). A Brown, Boveri and Co. (BBC) Model SE120 dual pen, chart

recorder (Brown, Boveri and Co., Goerz/ Metrawatt, Vienna, Austria) was used for the recording of the chromatograms.

Chemicals

HPLC-grade methanol (MeOH) used in the mobile phase was obtained from EM Science (Gibbstown, NJ). ACS grade phosphoric acid was obtained from Baker (Phillipsburg, NJ). ACS grade sodium hydrogen phosphates (monobasic and dibasic) were obtained from Sigma (St Louis, MO). ACS grade sodium octyl sulphate (SOS) was obtained from Aldrich (Milwaukee, WI). Deionized water was prepared in our laboratory using a Barnstead Water purification system (Sybron Corp., Boston, MA). Human citrated plasma was obtained from Sigma. Peptide 520 was obtained from Genentech, Inc. (South San Francisco, CA). All chemicals were used as received without further purification.

Chromatographic and EC detection conditions

The mobile phase used for elution was a methanol–phosphate buffer (23:77, v/v) solution. The phosphate buffer was prepared by dissolving 3.0 g Na_2HPO_4 and 7.6 g NaH_2PO_4, and 0.233 g SOS in 1 l deionized water. The buffer was adjusted to pH 6.5 and filtered through a Millipore HAWP 0.45 μm filter (Millipore, Bedford, MA). All LC solvents were degassed under vacuum with stirring before use. The flow rate was 1.0 ml min^{-1}. The applied potential for the determinations was +1.0 V. The potentials for the hydrodynamic voltammetry of peptide 520 were varied from +0.20 to +1.20 V. The glassy carbon working electrodes were polished before use each day.

Sample preparation

Human plasma was dissolved in deionized water and the concentration of the plasma solution was 20 mg ml^{-1}. All plasma solutions were filtered by a 0.45 μm, Aodisc syringe filter (Supelco, Bellefonte, PA). Different concentrations of peptide 520 were injected into a series of plastic test tubes, and then 0.5 ml human plasma solution was added to make a series of concentrations of peptide 520 as 10 mg ml^{-1} human plasma. The standard solutions of peptide 520 were prepared by diluting 10 mg ml^{-1} peptide 520 in the blank or in deionized water. In the recovery studies, 10 ppm levels were prepared in human plasma solution, as above. These were then directly injected into the HPLC–UV system, and peak areas were used with an external calibration plot to determine per cent recovery (n = 3).

Results and Discussion

The determination of peptide 520 by RPLC–UV/hv–EC

Photochemical derivatization for improving detection of the peptide called '520', which was provided by Genentech, has been investigated. This peptide has five amino acids in a cyclic (ring-structure) arrangement and one extra amino acid at the C-terminal. Its structure shows no EC active amino acids, such as aromatic or thiol functional groups. However, one part of this peptide, R-CH_2-S-CH_2-R′, is EC active after photolysis (hv), and thus the overall peptide can be determined by hv–EC detection at mild oxidative potentials.

Experiments were performed by using RPLC–UV/hv–EC and an on-line photolysis unit. A photolytic derivatization could be made by switching the lamp on. The detectability of the peptide was made possible by using light as a derivatization 'reagent' (Fig. 1). The lowest detection limit was 500 ppb. It was obvious when the lamp was turned on that the noise became larger. Thus, the baseline noise limited the lower detection limit of the peptide. In previous publications, we have described the theory, instrumental operations, and numerous applications of using photolytic–EC derivatization approaches, post-column in HPLC modes [23–28]. The effectiveness of this detection scheme is dependent on the particular structure of the analyte. In this case, we are dealing with a peptide having no photolytically labile aromatic amino acids, such as tyrosine, phenylalanine, or tryptophan, all of which lead to improved EC detection in other peptides or proteins [42].

Comparison of chromatograms for UV and hv–EC detection for the determination of peptide 520 in human plasma

In comparing the two chromatograms for peptide 520 in human plasma by hv–EC and UV, good separations from human plasma matrix peaks were achieved (Figs 2 and 3). In each detector chromatogram, there were no interfering peaks present in the blanks, and a single peak appeared at about 21 min for the peptide of interest. Each detector led to

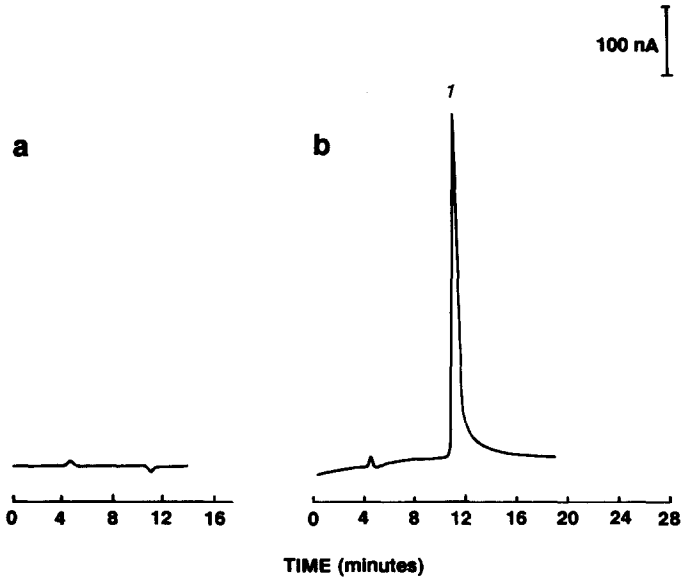

Figure 1
Chromatograms of peptide 520 determined by on-line, post-column photolysis RPLC–EC. Conditions: column: LiChrocart C_{18}, 250 × 4 mm; mobile phase: 15% MeOH + 85% buffer; buffer: 3 g Na_2HPO_4 + 7.6 g NaH_2PO_4 + 1 l H_2O; flow rate: 1.0 ml min^{-1}; detection: EC at +1.0 V, 200 nA, dual glassy carbon working electrode, Ag–AgCl reference electrode. Peak no. 1 is peptide 520: (a) lamp off; (b) lamp on.

acceptable peak shapes for the peptide. Increasing concentrations injected led to concomitant increases in the peak height of this one peak. A buffer pH of 6.5 was selected because the pI of peptide 520 is 6.5. The retention time of peptide 520 can be controlled by adjusting the organic component of the mobile phase. A methanol concentration of 22% was chosen because it afforded good separation from the matrix and minimum run time. There was no EC activity of the standard peptide in the absence of an initial photolytic derivatization step.

Recovery study of peptide 520

The recovery study of peptide 520 spiked into human plasma, which was determined by UV, showed a quantitative recovery (103 ± 1.2% RSD) of the peptide spiking (n = 3). This demonstrated that peptide 520 did not bind to proteins in human plasma, when directly spiked into plasma. Because this study involved a simple spiking of human plasma with the peptide standard, rather than drawing biofluids from an animal or patient given the peptide as a drug regimen, per cent recovery need not apply to an *in vivo* study. It is still conceivable that even with *in vivo*, metabolic type samples, a high per cent recovery will be realized by direct injection of biofluids into the HPLC system.

Linearity of peptide 520 in human plasma

The linearity of peptide 520 from 0.5 to 25 ppm concentrations in human plasma was very good for both the UV and hv–EC detectors (r^2 = 0.9999 for UV and r^2 = 0.999 for hv–EC). The detection limit of peptide 520 in human plasma was 200 ppb (0.2 μg ml^{-1}) for UV and 500 ppb for hv–EC. This was because the photolysis step increased the noise of the baseline. In Table 1, the RSDs are listed for peptide 520 in human plasma for the linearity studies. These linearities indicated that calibration plots with external standards could conceivably be used for quantification studies, if matrix matching plots were developed in human plasma. However, a standard additions method was eventually utilized, since this avoided all matrix associated problems, did not require separate matrix matched calibration plots, and was simple to employ.

Hydrodynamic voltammogram (HDV) of peptide 520

The HDV for peptide 520 is shown in Fig. 4. As can be seen, the hv–EC response dramatically enhanced as the potential neared +1.0 V. However, the increase was less dramatic when the potential was above +1.0 V, and the baseline noise was greatly increased. For these reasons, a potential of +1.0 V was chosen for the analysis of peptide

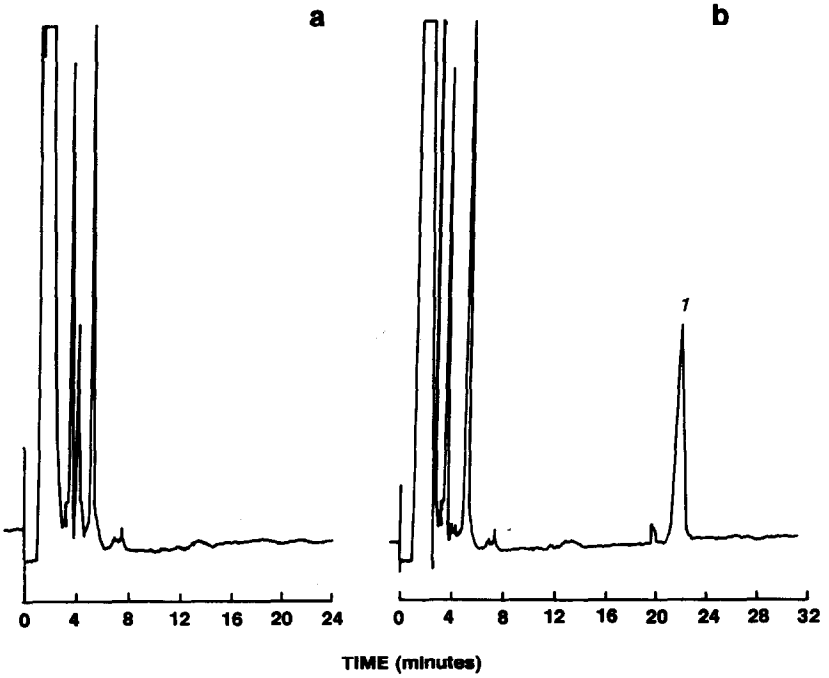

Figure 2
Chromatograms of peptide 520 in human plasma determined by RPLC–UV. Conditions: column: Supelco LC18-DB, 150 × 4 mm; mobile phase: 22% MeOH + 78% buffer; buffer: 3 g Na_2HPO_4 + 7.6 g NaH_2PO_4 + 0.232 g SOS + 1 l H_2O, pH 6.5 adjusted by phosphoric acid; flow rate: 1.0 ml min^{-1}; detection: UV at 210 nm, S = 2. Peak no. 1 is peptide 520: (a) human plasma matrix; (b) human plasma spiked with 10 ppm peptide 520.

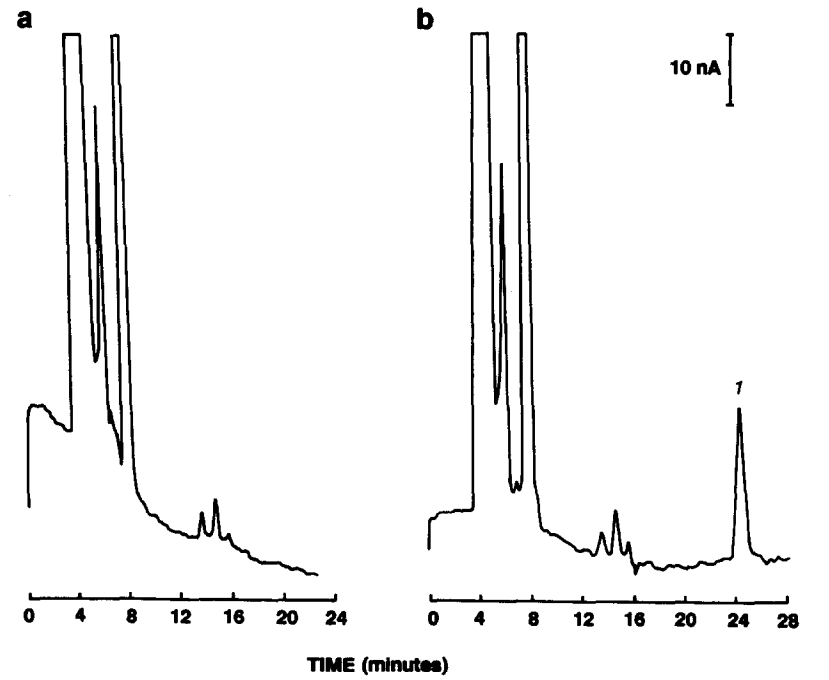

Figure 3
Chromatograms of peptide 520 in human plasma determined by RPLC–EC. See Fig. 2 for chromatographic conditions. Detection: EC at +1.0 V, 10 nA, glassy carbon working electrode, Ag–AgCl reference. Peak no. 1 is peptide 520: (a) human plasma matrix; (b) human plasma spiked with 10 ppm peptide 520.

Table 1
Relative standard deviation of UV and hv–EC response measurements in the linearity study*

Peptide 520 (ppm)	0.5	1	10	25
UV responses ± SD	0.8 ± 0	1.23 ± 0.03	7.23 ± 0.03	17.6 ± 02
RSD (%)	0	0.2	0.4	0.9
EC responses ± SD (nA)	0.07 ± 0	0.13 ± 0.01	1.01 ± 0.02	2.67 ± 0.6
RSD (%)	0	4.6	2	2

* $n = 3$ for each sample; the responses were measured in peak heights. See Fig. 2 for chromatographic conditions. All peptides were in human plasma.

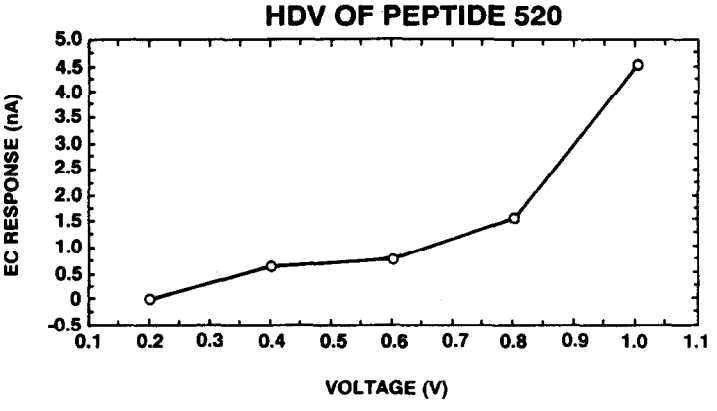

Figure 4
HDV of 10 ppm peptide 520. See Fig. 2 for chromatographic conditions. Detection: EC at 10 nA, dual glassy carbon working electrode, Ag–AgCl reference.

520. Lower operating potentials would have significantly decreased the detector signal response, leading to higher detection limits and poorer sensitivities. Operating at >+1.0 V would have led to a worse signal-to-noise ratio, and again higher detection limits and poorer sensitivities. The HDV, Fig. 4, became horizontal and flat above +1.0 V, showing no signal increase above that at +1.0 V.

Effect of photolytic sources

A Zn lamp was used as another irradiation source to photolyse peptide 520, in order to improve the hv–EC sensitivity [37]. However, the results were inferior, in terms of peak height and shape, for this peptide, when compared with a Hg lamp. We have previously presented similar results on the comparative performances of Zn vs Hg lamps in post-column, photolytic reaction schemes [25]. Because knitted open tubular (KOT) photolytic reactors must be made of a flexible material, usually PTFE (polytetrafluoroethylene, Teflon), transparency below 254 nm is generally poor, especially at the maximum output of a Zn lamp, *ca* 214 nm. Hence, a quartz, coiled tube must be used as the reactor, which adds significant bandspreading and a loss of chromatographic performance. Peak shapes generally become broader with a coiled reactor, as opposed to the knitted reactor with PTFE materials. Peak height also is decreased, efficiencies (plate counts) are decreased, and minimum detection limits are increased, not decreased, in going from a Hg lamp with a KOT reactor to a Zn lamp with a coiled quartz reactor. We have previously summarized the loss of chromatographic performance, and also quantitated the degree of inferior results in such comparisons. In general, a Zn lamp with a quartz reactor coil will *always* produce inferior results, as evidenced by smaller peaks, unless there is no analyte absorbance with the Hg lamp whatsoever, but there is some with the Zn lamp. This is somewhat predictable.

Effects of different working electrodes

The principle of using dual gold–mercury (Au–Hg) electrodes in series for the determination of thiols and disulphides is that disulphides are reduced at the upstream electrode. Then, thiols and reduced disulphides are detected oxidatively downstream at the second Au–Hg electrode, at a potential of +0.10 V vs Ag–AgCl [12]. The on-line, post-column, photolysis irradiator was developed to

replace the Au–Hg upstream electrode in order to reduce disulphides [37]. A single, smooth, mirror-like Au–Hg working electrode was made to measure peptide 520 in human plasma. A very good baseline was obtained when the potential was set at +0.15 V vs a Ag–AgCl reference electrode. However, the sensitivity for peptide 520 at the Au–Hg working electrode was not as good as that at a glassy carbon electrode. Peak height responses, at the same concentrations injected on each separate electrode, consistently showed smaller peak heights for the Au–Hg. Even varying the working potentials over a broad range with each electrode, did not improve the response from the Au–Hg type.

In general, Au–Hg type electrodes are useful for those functional groups, such as thiols, that can first complex with the Hg to form organometal species readily oxidized. However, with this particular peptide, containing no free thiol groups, there is no other functional group that could complex or react with the Au–Hg surface prior to EC reactions. It is not surprising that there was no improvement in peptide sensitivity (lower detectability) for this particular peptide in going to the Au–Hg type working electrode material. Other peptides, structure dependent, might show improved responses with alternate working electrodes or chemically modified electrode materials. We are currently investigating such possibilities and opportunities.

Single blind spiking of peptide 520 in human plasma

Single blind spiked samples of peptide 520 were determined with the glassy carbon working electrode and UV detector. There were three unknown concentrations, which were determined by the standard additions method (Table 2). The chromatographic conditions are indicated in Fig. 3. In general, there was acceptable agreement between the spiked/known levels and those determined by both UV and hv–EC detection methods. However, the relative errors determined by hv–EC were larger than those by UV. Perhaps this was caused by some species in human plasma adsorbing onto the working electrode surface, causing a change in its response pattern from injection-to-injection.

According to the Cottrell equation:

$$i = \frac{n \cdot F \cdot A \cdot D_0^{1/2} \cdot C_0^*}{\pi^{1/2} \cdot t^{1/2}} .$$

The response in EC detection (i = current) is a function of the effective area (A) of the working electrode. If there is adsorption on the electrode surface, it leads to a decrease of the effective area of an electrode and an increase in residual background current. In general, this is an irreversible procedure, which can affect the accuracy of EC detection. Detector fouling with biofluids is a common occurrence in LCEC, unless micellar mobile phase conditions are employed. This may be improved or alleviated by careful, repeated, and on-going surface cleaning and reactivation (polishing, laser ablation, etc.) [31–36]. In our studies, repeated surface polishing was employed, which may be less than ideal to reduce per cent relative errors in hv–EC.

Conclusions

In summary, we have shown that hv–EC detection is compatible with micellar HPLC for the determination of peptide analytes in human plasma. No initial sample cleanup or preparation has been required, but rather direct injection in HPLC is entirely feasible and practical. The higher detection limit of hv–EC compared to UV was due to the fact that the peptide contained only a thioether functionality, as opposed to an aromatic amino acid, disulphide or free thiol. Previously, we

Table 2
Single blind spiking of peptide 520 in human plasma*

Sample	Actual†	EC	%RSD‡	%RE§	UV	%RSD	%RE
A	3.75	3.64	2.8	3.0	3.67	1.1	2.1
B	7.5	8.4	3.9	12	7.58	1.4	1.1
C	22.5	25.3	6.1	12	23.8	2.9	5.8

*See Fig. 2 for chromatographic conditions.
†Levels in ppm.
‡n = 3.
§Per cent relative error = [actual level − determined level/actual level] × 100.

had shown that detection limits for thioethers (e.g. methionine) were higher than for aromatic, disulphide and thiol species by photolysis–EC [38]. Although UV detection offered lower detection limits for the particular peptide studied here, 200 vs 500 ppb, as well as better reproducibility and quantitation, we expect that those peptides which contain aromatic, disulphide and thiol species will provide lower (better) detection limits than UV, even in biofluids. Dual electrode response ratios, though not utilized here, even in the hv–EC mode, can often provide improved analyte identification when compared with an authentic standard, far and above what UV detection usually provides, perhaps especially for peptides [43].

Polishing and cleaning working electrodes are very important steps, because these can eliminate adsorption of species present in human plasma onto the electrodes and will, overall, increase the reproducibility of determinations. This is clearly a shortcoming of the basic EC approach for biofluids, especially when, as with peptide 520, UV detection offers some advantages. Pulsed amperometric detection (PAD) methods may provide improved reproducibility and stability of the working electrode for drugs/peptides in biofluids, though not attempted here. It is also true that using conventional GCE working electrodes without PAD waveforms, conventional amperometry, may not be readily applicable to routine, automated analyses for biofluids. Chemically modified electrodes (CMEs) or membrane-bound working electrodes might also offer stability and reproducibility advantages.

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