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QUANTITATIVE LIQUID CHROMATOGRAPHIC DETERMINATION OF DISULFIDE-CONTAINING PEPTIDE ANALOGUES OF VASOPRESSIN WITH DUAL Hg/Au ELECTROCHEMICAL DETECTION

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SUMMARY

Quantitative methodology was developed for the analysis of disulfide-containing peptide analogues of vasopressin in biologic media. The procedure employs sample clean-up by an ion-exchange solid-phase extraction cartridge, followed by high-performance liquid chromatography with electrochemical detection. The detector is a dual Hg/Au system operated in series in which the disulfide-containing peptides are first reduced at the upstream electrode and then detected as free thiols at the downstream electrode. The assay is linear in the range 2–100 ng/ml (approximately 2–100 pmol/ml) of urine with a lower limit of detection of 1 ng ($\sim$1 pmol) on column. The method displayed general utility for a number of structural analogues of vasopressin.

INTRODUCTION

There is an increasing interest in the use of synthetic analogues of naturally occurring peptide hormones as a basis for designing novel therapeutic agents. One area that is currently under active investigation is the development of antagonists of arginine vasopressin (AVP, Fig. 1), a nonapeptide that mediates the reabsorption of water by the kidney [1,2]. A number of agents that have been developed are structural analogues of this hormone, and contain a cyclized hexapeptide ring system joined by a disulfide bridge. The octapeptide [pentamethylene propionyl $(PMP)_1$-D-Tyr$(C_2H_5)_2$-Val_4-desGly]-AVP (SK&F 101926, I, Fig. 1) is one of the most potent V_2-receptor antagonists discovered to date and is currently undergoing clinical trials.

The development of a specific, rapid assay for I and its analogues in biological matrices is an essential prerequisite to carrying out detailed pharmacokinetic

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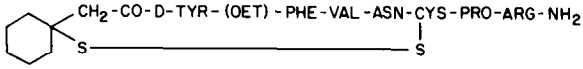
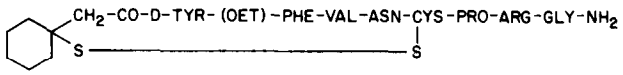
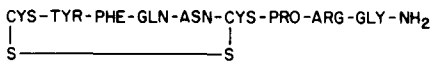
SK&F 101926 (I)



SK&F 101498 (INTERNAL STANDARD)



VASOPRESSIN



OXYTOCIN

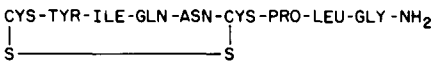


Fig. 1. Structures of vasopressin analogues.

studies on these agents. The problem of developing a generally useful assay for this class of compounds is complicated by their high potency, where therapeutic doses in the range 10–100 μg/kg are typical. A practical assay, therefore, must have a useful detection limit in the 0.1–10 pmol/ml range. The structure of I is characterized by the lack of an N-terminus, a blocked C-terminus and a series of hydrophobic or relatively unreactive residues. The inherent absorption or fluorescence characteristics of this structure are insufficient to achieve the required degree of sensitivity, and the use of conventional absorbance or fluorescence derivatizing agents is ruled out by the lack of a suitably reactive functional group. However, one structural feature that does provide a possible basis for quantification is the disulfide bond.

Prior studies have demonstrated the feasibility of disulfide analysis by high-performance liquid chromatography (HPLC) with post-column electrochemical reduction and subsequent electrochemical determination of the free thiol. Eggli and Asper [3] employed a silver amalgam grain-packed electrode to quantitatively reduce cystine eluting from the HPLC column. The resulting cysteine was quantified electrochemically at the mercury pool electrode. Allison and Shoup [4] described a similar dual-electrode approach in which two gold amalgam electrodes were employed in series. The upstream electrode (generator) was set at a negative potential of -1.0 V versus Ag/AgCl for the reduction of disulfides (reaction 1) and the downstream electrode (detector) was set at $+0.15$ V for monitoring the resultant free thiol reaction (reaction 2):

$$RSSR + 2H^+ + 2e^- \rightleftharpoons 2RSH \quad (1)$$

$$2RSH + Hg \rightleftharpoons Hg(RS)_2 + 2H^+ + 2e^- \quad (2)$$

The low potential of the downstream electrode coupled with the prior reduction step provided a remarkably specific detector for disulfides. Allison et al. [5] later demonstrated the utility of this system for the simultaneous determination of cysteine, glutathione, pencillamine and their respective disulfides. The present study was undertaken to explore the feasibility of employing the dual Hg/Au cell in the analysis of disulfide-containing analogues of vasopressin.

EXPERIMENTAL

Chemicals

Vasopressin and oxytocin were purchased from Vega Biochemicals (Tucson, AZ, U.S.A.). I (SK&F 101926, purity > 98%) and internal standard (I.S., SK&F 101498, purity > 98%) were provided by the Peptide Chemistry group, Smith Kline and French Labs. (Swedeland, PA, U.S.A.). Monochloroacetic acid was purchased from Mallinckrodt (Boston, MA, U.S.A.) and HPLC-grade acetonitrile was obtained from Baker (Philadelphia, PA, U.S.A.). All other chemicals were reagent grade or equivalent and were obtained from local sources.

Apparatus

A DuPont Instrument Series 8800 chromatographic pump (DuPont Instruments, Wilmington, DE, U.S.A.) and a Bioanalytical Systems LC-4B dual Hg/Au detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) were used for all analyses. An SSI Lo-Pulse dampener (Scientific Systems, State College, PA, U.S.A.) was placed between the pump and automatic injector (Wisp 710B; Waters-Millipore, Milford, MA, U.S.A.) to reduce baseline cell noise. PTFE tubing throughout the system was replaced with stainless-steel tubing to exclude oxygen. An Aquapore RP300 C_8 HPLC column (25 × 0.46 cm, 10 μm particle size; Brownlee Labs., Santa Clara, CA, U.S.A.) was used for all analyses at a flow-rate of 1.0 ml/min. The detector output signal was interfaced to a CIS laboratory information management system (Beckman/Computer Inquiry Systems, Paramus, NJ, U.S.A.).

Chromatographic conditions

All mobile phases were prepared with deionized water and filtered through a 0.2-μm Nylon 66 filter (Rainin Instruments, Woburn, MA, U.S.A.). Mobile phase was purged with helium continuously to remove dissolved oxygen. The mobile phase was an isocratic system containing 33% acetonitrile and 67% 0.1 *M* monochloroacetic acid buffer (pH 3.0) with 0.25% (v/v) trifluoroacetic acid (TFA) for analysis of I and I.S. Vasopressin and oxytocin were analyzed with similar solvent systems containing 14 and 20% (v/v) acetonitrile, respectively. The dual series Hg/Au cells were prepared as previously described [4]. The electrodes were arranged in series with the upstream electrode held at −1.0 V and the downstream electrode, where the column effluent was monitored, set at +0.15 V versus the Ag/AgCl reference electrode. The controller output was used at a sensitivity

setting of 1 nA f.s. and a time constant of 2.0 s for all analyses unless otherwise noted.

Standard solutions

Stock standard solutions were prepared for I, I.S. ([PMP_1-D-Tyr(C_2H_5)$_2$-Val_4]-AVP, Fig. 1), vasopressin and oxytocin at 1 mg/ml in absolute ethanol. All stock solutions were stored at 4°C and kept for a maximum of three weeks. Dilutions of the stock solutions for the standard curve determinations were prepared and used on the day of analysis. The standard curve for I and its internal standard was constructed by spiking blank, human urine with known concentrations of I over the range 2–100 ng/ml, plus 50 ng/ml of internal standard. Each urine sample was then extracted as described below. Standard curves for vasopressin and oxytocin were prepared from dilutions of standard stock solutions over the range 25–100 ng/ml in absolute ethanol. Hydrodynamic voltammograms were constructed for all four compounds using standard solutions at a concentration of 150 ng per 5 μl for vasopressin and 200 ng per 5 μl for the others. A 5-μl aliquot was injected onto the column for all voltammogram determinations.

Sample preparation

Carboxylic acid cation-exchange solid-phase extraction cartridges (1 ml Bond-Elut CBA; Analytichem International, Harbor City, CA, U.S.A.) were prepared by washing with 1 ml of hexane, followed by 1 ml methanol and 1 ml water. A 10-ml aliquot of human urine was passed through the CBA cartridge, which was then washed with two 1-ml aliquots of water followed by 100 μl of methanol; the column was then air-dried for 3 min. I and its internal standard were eluted from the column with four 100-μl aliquots of 1% TFA in methanol. This eluent was evaporated to dryness under nitrogen gas, redissolved in 100 μl of 1% TFA in methanol and transferred to a WISP limited-volume insert. A 25.0-μl aliquot of each sample was injected directly onto the HPLC column for analysis.

Precision, accuracy, linearity and recovery

The assay was validated by replicate analyses ($n=5$) of human urine containing I at concentrations of 10, 50 and 90 ng/ml. The replicate analyses were repeated on each of three days. Recovery was calculated by comparison of the peak areas of the extracted standards with the peak areas of the unextracted standards (in methanol) which were injected directly on-column.

Quantification

Urine samples were processed according to the procedure described above. The concentration of I was determined from the standard calibration curve run on the same day. Calibration curves were constructed by plotting peak-height ratios of I to the internal standard versus concentration of I in urine and fitting the data with a linear regression.

RESULTS AND DISCUSSION

Quantitative analytical methodology was developed for the vasopressin analogue I in human urine. A related structural analogue of vasopressin was used as

internal standard. The peptides were isolated from urine by a one-step solid-phase extraction procedure and analyzed by HPLC using the dual series Hg/Au cell as a combination post-column reactor and detector. The methodology that is described contains several novel features. As with most assays that involve biological media, the procedure used for sample extraction and clean-up is an important factor. A rapid single-step extraction procedure was developed using an ion-exchange-based solid-phase disposable cartridge. This results in a relatively pure extract that can then be analyzed directly by HPLC. Disulfide reduction was readily accomplished at the first electrode of the in-line dual cell thus avoiding the use of complex off-line procedures for chemical reduction. Specificity, in addition to that provided by selective sample clean-up, was introduced through use of a second Hg/Au electrode which provides remarkable selectivity for thiol-containing compounds, due to the low, but optimum, oxidation potential required for thiol-catalyzed mercury oxidation. The Hg/Au cell also affords optimum sensitivity since the low operating potential results in low background current and minimal baseline noise.

Extraction and chromatography

I and the internal standard are efficiently separated from urine with a weak cation-exchange cartridge. Although the elution of I from the cartridge could be accomplished with methanol, volumes in excess of 4 ml were required for quantitative recovery. Methanol–hydrochloric acid provided excellent recovery but also eluted interfering substances. Methanol–TFA afforded recoveries in excess of 90% using only 400 μl of eluent and did not introduce substances that interfered with the chromatography. Recovery of I from urine following the solid-phase extraction, evaporation and transfer into autosampler vials was $74 \pm 4\%$ (mean $\pm$ S.D.).

Fig. 2 illustrates a chromatogram of I and internal standard after extraction from human urine. The two components are well resolved under these conditions. Slight changes ($< 1\%$) in the organic content of the mobile phase dramatically altered k' (capacity factor) values for the analytes. Under isocratic conditions of 33% acetonitrile the dual cell detector operated in a stable and reproducible fashion. The use of even moderate gradients produced marked baseline changes which rendered use of the cell impractical in the 1-nA range. However, in the 10-nA range gradient elution (1%/min; 30–60% acetonitrile) results in tolerable baseline changes. Under these gradient conditions the minimum detection limits for vasopressin analogues was in the range 20–50 ng ($\sim$20–50 pmol) injected on-column.

During preliminary studies, a mobile phase consisting of acetonitrile–water containing 0.25% (v/v) TFA was investigated. With this solvent system the baseline noise prevented the attainment of detection limits in the 1–10 ng ($\sim$1–10 pmol) range. The addition of monochloroacetate buffer (0.1 *M*) to the mobile phase provided markedly reduced baseline noise and resulted in optimized detection limits ($\sim$1 pmol on column). When TFA was omitted from the mobile phase substantial peak broadening occurred.

To maintain a relatively noise-free baseline with minimal offset current it is

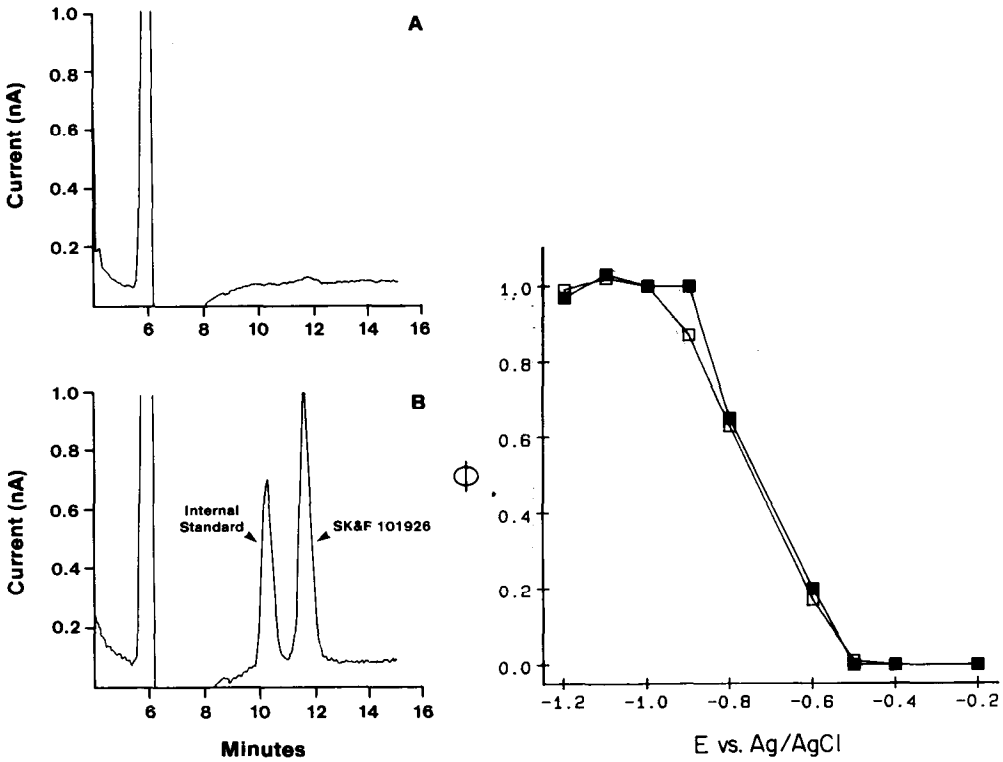


Fig. 2. Dual-electrode (Hg/Au) chromatograms of (A) blank urine and (B) urine containing I (SK&F 101926) (100 ng/ml) and internal standard (50 ng/ml). A urine extract was prepared by solid-phase extraction as described in Experimental. Internal standard and I have retention times of approximately 10.5 and 12.2 min, respectively. No interfering substances were detected in blank urine.

Fig. 3. Hydrodynamic voltammograms of I (□) and internal standard (■) on the Hg/Au electrodes. Peak-height responses at electrode 2 (+0.15 V) as a function of applied potential at electrode 1. Each point represents the mean of two injections of 200 ng of either I or internal standard. ϕ = peak current/peak current at −1.0 V.

essential that precautions be taken to remove oxygen from the mobile phase. This was accomplished by helium sparging the mobile phase and by replacing all PTFE tubing with stainless-steel tubing. In addition, oxygen dissolved in the sample produced a significant negative signal after injection (Fig. 2, 6–8 min). This signal was eliminated by degassing the sample prior to injection. However, degassing of small volumes can result in a substantial loss of solvent and was therefore not routinely undertaken in these studies. Retention times of the analytes were adjusted to avoid interference from the sample associated oxygen signal.

Optimization of electrode potentials

The reduction potential required for cleavage of the disulfide bond was determined for both the internal standard and I. The potential at the downstream electrode was maintained at +0.15 V while the upstream electrode was varied from −0.2 to −1.2 V. The hydrodynamic voltammograms are shown in Fig. 3.

TABLE I

WITHIN- AND BETWEEN-DAY PRECISION AND ACCURACY DATA FOR THE ANALYSIS OF I IN URINE

The assay was validated by replicate analyses ($n = 5$) of human urine containing I at concentrations of 10, 50 and 90 ng/ml. The replicate analyses were repeated on each of three days.

Theoretical concentration (ng/ml)	Day No.	Analytical concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (C.V.) (%)	Mean C ratio*	Bias**	Within-day C.V. (%)	Between-day C.V. (%)
10	1	9.67 ± 0.60	6.20	97	−0.33	5.30	5.98
	2	10.68 ± 0.58	5.43	107	0.68		
	3	10.80 ± 0.46	4.26	108	0.80		
50	1	46.74 ± 1.24	2.65	94	−3.26	2.66	5.82
	2	48.66 ± 1.33	2.73	97	−1.34		
	3	52.38 ± 1.36	2.60	105	2.38		
90	1	77.12 ± 4.09	5.30	86	−12.88	5.40	10.60
	2	94.23 ± 5.28	5.60	105	4.23		
	3	92.03 ± 4.87	5.29	102	2.03		

*Ratio of mean analytical concentration to theoretical concentration × 100.
**Mean analytical concentration − theoretical concentration.

Both I and the internal standard display plateaus at approximately −1.0 V. Therefore a potential of −1.0 V was used at the upstream electrode in all quantitative studies. The potential for the downstream electrode was set at +0.15 V which is the previously established optimum for thiol-catalyzed mercury oxidation [4]. In order to maintain adequate sensitivity for the concentration range studied the surface of the electrodes (both upstream and downstream) was polished and a fresh layer of mercury reapplied once a week.

The extreme negative potential of −1.0 V required to reduce the disulfide bonds of the vasopressin analogues produced background currents of several hundred nanoamperes as well as excessive baseline noise. Therefore quantitative monitoring of the reduction step was not a practical approach to the trace analysis of these disulfides. However, at the downstream electrode where a potential of +0.15 V was applied a background current of less than 3 nA was routinely observed.

Linearity, precision and accuracy

The assay for I was linear in the range 2–100 ng/ml (~2–100 pmol/ml) urine (correlation coefficient 0.999). Concentrations in excess of 100 ng/ml urine were not studied. The within-day precision of the assay was 5.30% at 10 ng/ml, 2.66% at 50 ng/ml and 5.40% at 90 ng/ml (Table I). Between-day precision was 5.98, 5.82 and 10.60%, respectively. The accuracy of the assay expressed as (the mean observed concentration/expected concentration) × 100 was 86–108% (Table I).

Sensitivity

The lowest quantifiable concentration of I in urine was 2 ng/ml (~2 pmol/ml). The limit of detection for I with a signal-to-noise ratio of 2:1 was 1 ng (~1 pmol) injected on-column (detector settings: 1 nA f.s., time constant 2.0 s and injection

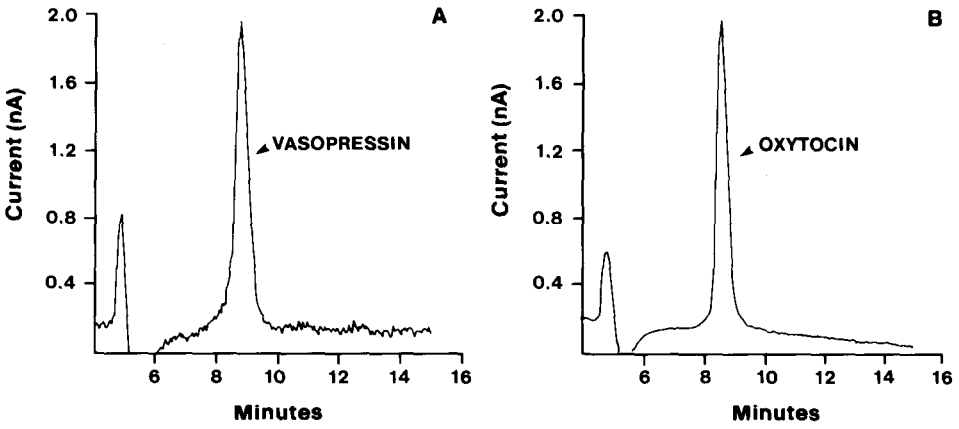


Fig. 4. Dual-electrode (Hg/Au) chromatograms of standard solutions of (A) vasopressin (200 ng) and (B) oxytocin (200 ng) in ethanol. Compounds were eluted under isocratic conditions with acetonitrile–0.1 *M* monochloroacetic acid (pH 3.0) containig 0.25% (v/v) TFA (14:86 for vasopressin and 20:80 for oxytocin).

volume 5 μl). It is possible that a substantial increase in sensitivity could be obtained by use of smaller I.D. columns (1–2 mm). Preliminary experiments using a 2 mm I.D. RP300 column gave approximately a four- to five-fold increase in signal-to-noise ratio for I. The total volume of the dual-electrode electrochemical cell as used in this assay is 5–10 μl and does not result in significant band broadening when used with 2 mm I.D. columns.

Specificity

The specificity of the assay was determined by analyzing blank urine and by analyzing urine known to contain metabolites of I. A chromatogram of blank urine is shown in Fig. 2. No interfering endogenous substances were observed. These chromatographic conditions will adequately separate I from its des-amido hydrolysis product, SK&F 102399 {[PMP-D-Tyr(OEt)-Phe-Val-Asn-Cys]-Pro-Arg-OH}. This is the only known urinary metabolite with a similar retention time to I (unpublished results).

Additional peptides

The suitability of the dual Hg/Au cell for analysis of vasopressin and a related peptide, oxytocin, was also explored. Fig. 4 illustrates the HPLC profiles of both compounds using a monochloroacetate–acetonitrile mobile phase. Hydrodynamic voltammograms, constructed by monitoring the downstream electrode (+0.15 V) while the upstream electrode potential was varied from −0.2 to −1.2 V, demonstrated that −1.0 V was suitable for reduction of the disulfide bonds (Fig. 5). Using electrode potentials of −1.0 V and +0.15 V for the upstream and downstream electrodes, respectively, vasopressin displayed a linear response in the range 25–200 ng (~25–200 pmol) on-column (correlation coefficient 0.997). The lower limit of detection for vasopressin and oxytocin was 1 ng (~1 pmol) on-column with a signal-to-noise ratio of 3:1.

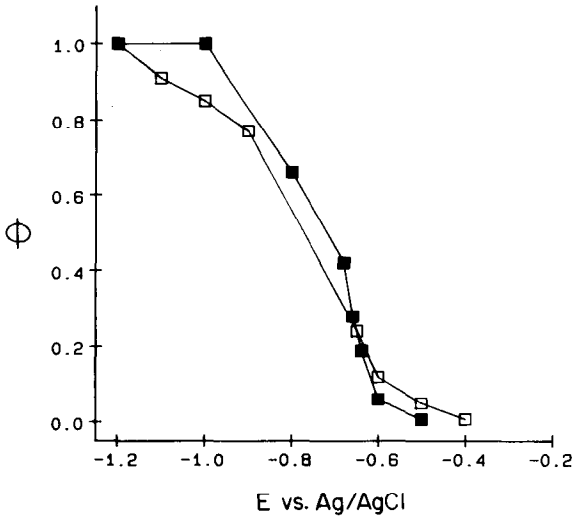


Fig. 5. Hydrodynamic voltammograms of vasopressin (□) and oxytocin (■) on the Hg/Au electrodes. Peak-height responses at electrode 2 (+0.15 V) versus Ag/AgCl as a function of applied potential at electrode 1. Each point represents the mean of two injections of either 150 ng of vasopressin or 200 ng of oxytocin. ϕ = peak current/peak current at −1.0 V.

Each of the vasopressin analogues, I, I.S., oxytocin and vasopressin displayed similar lower limits of detection of approximately 1 pmol on-column using the dual Hg/Au electrode cell. These detection limits are similar to those reported by Allison et al. [5] for glutathione disulfide (5.7 pmol) using the dual Hg/Au cell and by Eggli and Asper [3] for cystine (0.5 pmol) using a silver amalgam electrode for reduction and a mercury pool electrode for detection.

The optimum potential for the reduction of the disulfide bonds of the vasopressin analogues was −1.0 V. An optimum reduction potential of −1.0 V was also reported both by Allison et al. [5] for glutathione disulfide and by Lazure et al. [6] for vasopressin and atrial natriuretic factor.

CONCLUSIONS

These results demonstrate the general utility of the dual Hg/Au electrochemical detector for the quantitative determination of disulfide-containing peptide analogues of vasopressin. This methodology is particularly useful in instances where the analyte does not contain reactive functional groups that are suitable for derivatization with conventional absorbance or fluorescence 'tags'. The low volume of the dual-electrode flow cell (5–10 μl) is compatible with difficult chromatographic separations where extra-column contributions to band broadening must be minimized. The specificity of the detector when coupled with the selectivity of an ion-exchange solid-phase clean-up provided a remarkably clean chromatogram from biological media. This work represents the first reported method using HPLC with electrochemical detection for the quantitative determination of disulfides in cyclic peptide molecules.

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