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High-performance liquid chromatographic determination of sulfated peptides in human hemofiltrate using a radioactivity monitor

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Abstract

Specific labeling of tyrosine sulfate-containing peptides was achieved using a differential iodination approach. In a complex peptide mixture from human hemofiltrate, cold iodination to saturate free iodine binding sites was followed by mild acidic desulfation of tyrosine sulfate and subsequent radioiodination using iodine-125. Reaction steps were controlled by amino acid analysis using *o*-phthaldialdehyde precolumn derivatization and by spiking with a sulfated cholecystokinin fragment (CCK4-S). Separation of the peptide mixture with RP-HPLC on a C_{18} column coupled to a radioactivity monitor led to the sensitive (≤ 5 p*M*) and specific determination of tyrosine sulfate-containing peptides.

1. Introduction

Most known peptides containing tyrosine sulfate are constitutive and regulatory peptides like cholecystokinin (CCK), gastrin, secretogranin I and II and leu-enkephalin [1–4]. The specific labeling of tyrosine sulfate-containing peptides will enable the monitoring of metabolic activity and may yield in the quantification of regulatory processes.

A large variety of sulfated peptides is found in plasma. Animal cells also contain a high number of sulfated peptides and proteins as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis after incorporation of $^{32}SO_4$. Most of these proteins are not characterized [3]. Alkaline hydrolysis in combination with amino acid analysis has been used to determine sulfation of tyrosine in proteins and peptides in the picomolar range [5–7]. Similarly, a shift of peptide retention time resulting from desulfation by mild acid hydrolysis serves to verify the sulfation of a peptide [8]. The latter method can only be applied for known molecules with known retention times or relatively purified mixtures with only several peptides.

The method presented here is a new approach to facilitate the detection and subsequent quantification of sulfated peptides in crude biological mixtures. In this paper the new method is used for the first time in a plasma-like crude mixture.

Human hemofiltrate (HF) from patients with end-stage renal disease contains plasma-like concentrations of biologically active peptide hormones [cardiodilatin/atrial natriuretic peptide (CDD/ANP), guanylin, parathormone (PTH)] [9,15]. After peptide extraction using preparative anion-exchange chromatography, a concentrated

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mixture of peptides was obtained. Using this mixture, the determination of tyrosine sulfate was performed as shown by amino acid analysis with *o*-phthaldialdehyde (OPA) precolumn derivatization [10].

First amino acid analysis of tyrosine sulfate [5–8] was modified and led to quantitative determination of tyrosine sulfate in peptides. This was controlled by quantitative desulfation to tyrosine.

The chloramine-T method [11] was used to evaluate the specific labeling of sulfated tyrosine without hydrolysis of the peptides as described by Schepky et al. [12]. In brief, tyrosine residues and other possible iodine binding sites were saturated with cold iodine, then tyrosine sulfate was desulfated and subsequently labeled with radioiodine. The goal is to radiolabel only tyrosine residues which were previously sulfated. The difference in electron donor capacity of the aromatic ring enables the differentiation between tyrosine and tyrosine sulfate. This difference results in distinct changes in chemical reactivity. The presence of a sulfate group instead of the hydroxyl group reduces the reactivity, especially in the presence of reactants for electrophilic substitution. Electrophilic substitution in *ortho* position is strongly favored for tyrosine compared to tyrosine sulfate [13]. Iodination takes place via this electrophilic substitution in the iodination procedure using the chloramine-T method.

After 30 s iodination of free tyrosine was performed with high efficiency. Biiodination was completed after 240 s. Sequential radioiodination for 120 s showed incorporation of less than 7% of iodine-125 after 120 s. Free tyrosine sulfate was also iodinated using the chloramine-T method. Sequential radioiodination showed incorporation of iodine-125 of less than 5% at 120 s reaction time for both iodination steps.

The susceptibility of the amino acids arginine, cysteine, histidine, lysine, methionine, phenylalanine, serine and tryptophan for iodination and/or degradation by the chloramine-T method was evaluated at different incubation times. Tryptophan and methionine were degraded after 30 and 60 s, respectively. Final reaction products were not determined. Only basic amino acids showed a low amount of iodine incorporation (arginine 3%, histidine 5%), whereas the other amino acids were almost inert after 240 s total reaction time [12].

Detection of radiolabeled peptides from HF was performed using RP-HPLC coupled to a radioactivity monitor.

2. Experimental

2.1. Materials

$Na^{125}I$ was obtained from Amersham (Braunschweig, Germany). OPA and 0.133 *M* borate buffer were obtained from Hewlett-Packard (Waldbronn, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Sulfated cholecystokinin$_{26-29}$ (Asp–Tyr-[SO_3H]–Met–Gly–NH_2, CCK4-S) was from Saxon Biochemicals (Hannover, Germany).

2.2. Extraction of peptides from human hemofiltrate

A 2000-l volume of human hemofiltrate were extracted by preparative anion-exchange chromatography. After batchwise elution an ultrafiltration step with a molecular cutoff of M_r 20 000 was carried out for quantitative elimination of high-molecular-mass compounds. The ultrafiltrate was processed via anion-exchange liquid chromatography and eluted with increasing ammonium acetate concentrations. Fractions obtained were lyophilized and samples of 10–25 μg were used for determination of tyrosine sulfate.

2.3. Synthesis of tyrosine sulfate, sodium salt

Tyrosine sulfate was synthesized according to Penke and Nyerges [14] and yielded 73.4% of pure tyrosine sulfate. The purity of the product was controlled using capillary zone electropho-

resis, mass spectrometry and amino acid analysis.

2.4. Alkaline hydrolysis

Since tyrosine sulfate is degraded to tyrosine and sulfate using a conventional acid hydrolysis procedure (e.g. 6 *M* HCl, gas-phase hydrolysis for 2 h), alkaline hydrolysis was performed to preserve tyrosine sulfate.

Lyophilized fractions were subjected to alkaline hydrolysis with 0.2 *M* $Ba(OH)_2$ for 22 h at 110°C. Barium was eliminated by precipitation with 2 *M* H_2SO_4 at room temperature. The supernatant was neutralized with 1 *M* NaOH and lyophilized.

2.5. Dowex extraction

Samples were dissolved in 200 μl 0.1 *M* formic acid and applied to a pipette tip filled with 200 μl Dowex 50W-X2 cation exchanger (Serva, Heidelberg, Germany) previously washed with 3 volumes of 0.1 *M* formic acid. Unbound free tyrosine sulfate was recovered with 750 μl 0.1 *M* formic acid. The collected sample was adjusted to pH 5.5 with 1 *M* NaOH, lyophilized and dissolved in 25 μl of 0.4 *M* borate buffer pH 7.2. Finally tyrosine sulfate was measured using amino acid analysis as described below.

2.6. Amino acid analysis

Amino acid analysis was carried out on an amino acid analyzer (AminoQuant 1090, Series II, Hewlett-Packard). The samples (1 μl) were subjected to precolumn derivatization with OPA as described in the Hewlett-Packard instruction manual. Separation of amino acids was performed on an AminoQuant RP C_{18} column 200 × 2.1 mm (particle size 5 μm, Hewlett-Packard) with a gradient from 100% buffer A (20 m*M* sodium acetate, 0.018% triethylamine, pH 7.2, 0.3% tetrahydrofuran) to 60% buffer B (20 m*M* sodium acetate; pH 7.2, 40% acetonitrile, 40% methanol) in 17 min at 0.45 ml/min. Data were collected with the HP 9000 Series 300 data system.

2.7. RP-HPLC on a C_{18} column coupled to a radioactivity monitor

Separation of radioiodinated samples was performed on a Kontron 322 HPLC system with a HPLC 360 autosampler and a HPLC detector 742 (Kontron, Neufahrn, Germany) connected to a radioactivity monitor LB 506 C-1 with a γ-measuring cell J-1000 (volume: 100 μl; Berthold, Wildbad, Germany). Data acquisition was controlled by the LB 506 C-1 HPLC data system. Separation of analytes was performed on a 250 mm × 4 mm I.D. RP C_{18} column (particle size 5 μm, pore size 300 Å; Biotek, Östringen, Germany) at a flow-rate of 0.75 ml/min. The gradient was developed using 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile–water (80:20) (B): 0–50% B in 50 min; 50–100% B in 10 min; 100% B for 5 min; 100–0% B in 5 min. A 50-μl volume of each radioactive sample was loaded onto the column.

2.8. Saturation of tyrosine with iodine-127 (cold)

A 450-μl volume of 0.5 *M* sodium phosphate buffer (pH 7.5), 100 μl $Na^{127}I$ (285 mg/ml in phosphate buffer), 250 μl chloramine-T (10 mg/ml in phosphate buffer) and the samples dissolved in 100 μl phosphate buffer, all in an Eppendorf vial, were incubated at room temperature. After 120 s the reaction was stopped with 100 μl $Na_2S_2O_5$ (315 mg/ml in phosphate buffer).

2.9. Extraction with Sep-Pak cartridges

Sep-Pak C_{18} cartridges (Waters-Millipore, Eschborn, Germany) were preconditioned with 10 ml methanol followed by 20 ml of 10 m*M* HCl. After application of the sample iodinated with iodine-127 the cartridge was rinsed with 10 ml of 10 m*M* HCl to remove unbound material. Elution was carried out in two steps using 1 ml 30% isopropanol in 0.01 *M* HCl followed by 1 ml 50% isopropanol in 0.01 *M* HCl. The eluate was neutralized with 1 *M* NaOH and lyophilized.

2.10. Desulfation

In the desulfation process, lyophilized samples were dissolved in 20 μl 1 *M* HCl in a glass vial and incubated for 3 min in boiling water. The samples were then lyophilized.

2.11. Radioiodination

Samples were dissolved in 45 μl 0.5 *M* sodium phosphate buffer (pH 7.5) and added to an Eppendorf vial containing 10 μl 0.5 *M* sodium phosphate buffer (pH 7.5) with $2.7 \cdot 10^6$ cpm iodine-125, 25 μl chloramine-T (10 mg/ml in phosphate buffer). After incubation for 120 s, reaction was stopped by the addition of 10 μl $Na_2S_2O_5$ (315 mg/ml in phosphate buffer). Total assay volume was 90 μl. A 50-μl volume of the mixture was directly applied to the RP-HPLC system.

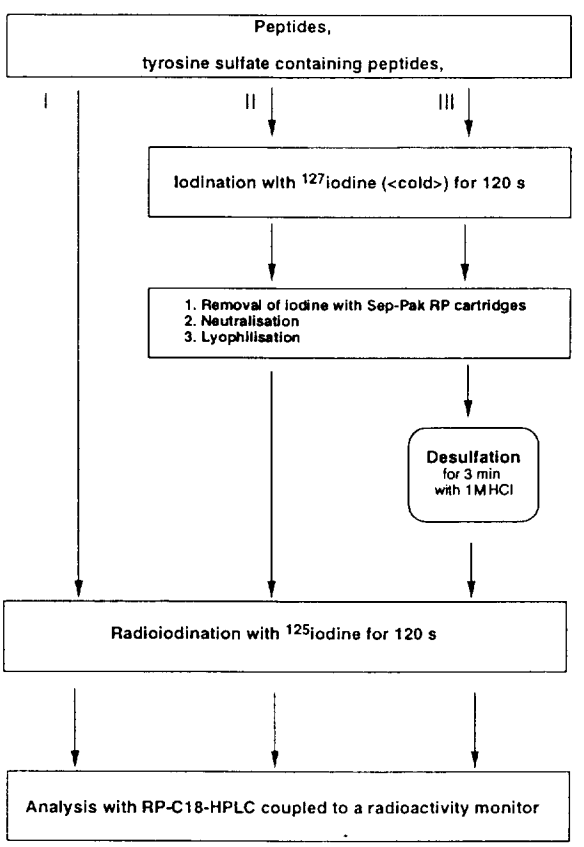

Fig. 1. Reaction scheme of differential iodination for determination of tyrosine sulfate.

2.12. Sequence for determination of sulfated peptides

The complete reaction sequence for determination of sulfated peptides is shown in the flow sheet (Fig. 1). The procedure can be divided into three main steps.

Step I. Sample is radioiodinated for 120 s.

Step II. Sample is first iodinated with iodine-127 for 120 s, followed by Sep-Pak extraction and a second radioiodination for 120 s.

Step III. Sample is desulfated with 1 *M* HCl after the first iodination, followed by Sep-Pak extraction and a subsequent second radioiodination for 120 s.

After radioiodination, samples are directly analyzed by RP-HPLC.

3. Results and discussion

In previous work we have shown that incorporation of radioactive iodine in synthetic peptides containing tyrosine sulfate was achieved [12]. Here we report the identification of tyrosine sulfate-containing peptides from a complex biological source.

3.1. Desulfation and amino acid analysis determination of tyrosine sulfate

Tyrosine sulfate (11 p*M*) and 1.5 p*M* tyrosine were mixed and subjected to amino acid analysis (Fig. 2). A clear separation of the two amino acids was achieved. After desulfation with 1 *M* HCl, no tyrosine sulfate but 12 p*M* tyrosine were detected (Fig. 2) (recovery 96%; $n = 5$). Desulfation was quantitative and lead to the generation of tyrosine readily detected with amino acid analysis. The generation of tyrosine proved the presence of tyrosine sulfate in the respective peak in amino acid analysis.

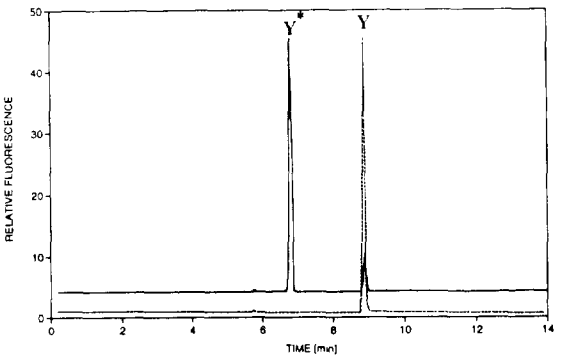

Fig. 2. Amino acid analysis with OPA precolumn derivatization. Solid line: 11 p*M* tyrosine sulfate and 1.5 p*M* tyrosine coinjected onto the column; Y* = tyrosine sulfate; Y = tyrosine. Broken line: 11 p*M* tyrosine sulfate and 1.5 p*M* tyrosine after desulfation; Y* = tyrosine sulfate; Y = tyrosine. Chromatographic conditions are described in the text.

3.2. Determination of tyrosine sulfate by amino acid analysis in complex peptide mixtures

To determine tyrosine sulfate in peptides, alkaline hydrolysis of samples followed by Dowex cation exchange was performed as described. Peptides from human HF were fractionated by anion exchange and aliquots of 10–25 μg were tested by amino acid analysis. In one fraction 5.9 p*M* tyrosine sulfate and 1.2 p*M* tyrosine could be determined (Fig. 3A). After desulfation of this sample a peak was detected eluting at the position of tyrosine sulfate (Fig. 3B). "Tyrosine sulfate" (1.19 p*M*) and 5.8 p*M* tyrosine could be detected. Either incomplete desulfation in this complex mixture or the occurrence of an additional substance coeluting with tyrosine sulfate could be the reason for this phenomenon. The real concentration of tyrosine sulfate may be calculated as the amount of tyrosine generated by desulfation. This additional approach is applicable in most cases, where tyrosine levels are kept low following Dowex extraction. The increase in tyrosine from 1.2 to 5.8 p*M* shows the conversion of the majority of tyrosine sulfate to tyrosine, thus proving the presence of tyrosine sulfate in this hemofiltrate peptide extract. In several hemofiltrate fractions no tyrosine sulfate could be detected. After desulfation procedure of these fractions no increased amount of tyrosine could be measured. Desulfation of tyrosine sulfate was shown to be complete when synthetic mixtures of tyrosine sulfate and tyrosine were used [12].

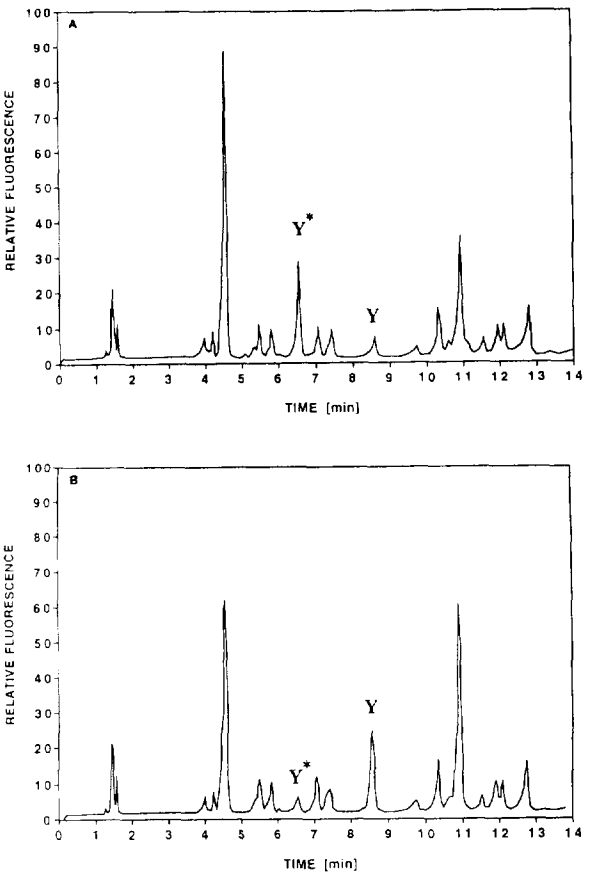

Fig. 3. Amino acid analysis with OPA precolumn derivatization. (A) A hemofiltrate fraction is injected onto the column after alkaline hydrolysis and Dowex cation-exchange extraction; Y* = tyrosine sulfate; Y = tyrosine. (B) The hemofiltrate fraction is treated as in (A) and then subjected to acidic desulfation; Y* = tyrosine sulfate; Y = tyrosine. Sample preparation and chromatographic conditions are described in the text.

3.3. Determination of endogenous sulfated peptides with differential iodination

The chloramine-T method [11] was used to evaluate the specific labeling of sulfated tyrosine without hydrolysis of the peptides as described by Schepky et al. [12]. In a first step (step I), an

aliquot of the sample was directly radioiodinated and separated using RP-HPLC on a C_{18} (data not shown). Incorporation of radioiodine results in a whole series of peaks as detected by radioactivity monitoring. This test is a good way of controlling the general labeling of our sample. Fig. 4A shows the chromatogram of the sample after first iodination with iodine-127 for 120 s, followed by Sep-Pak extraction and a second radioiodination for 120 s (step II). Two distinct peaks (Y and Z) can be seen eluting at high solvent concentrations and two minor peaks are found (X). Cold iodination does not seem to be quantitative since radioiodine is incorporated. Desulfation prior to radioiodination (step III) results in the appearance of an additional major peak at position X, whereas Y is practically unchanged and Z is increased. From Fig. 4A and B we conclude that X represents one or a few peptides containing tyrosine sulfate. After desulfation the strong increase in radioactivity suggests the presence of tyrosine sulfate converted to tyrosine by the desulfation procedure. The incorporated radioactivity is increased by a factor of 10. Peak Y shows no additional incorporation of radioiodine after desulfation and is therefore judged to contain little or no tyrosine sulfate. Peak Z also incorporates additional radioiodine after desulfation also suggesting the presence of tyrosine sulfate. At least two peaks were distinguishable in the chromatogram by increased incorporation of radioiodine after desulfation compared to the control HPLC.

These results show the necessity to perform two assays with the samples tested. First the degree of radioiodination without previous desulfation must be determined to obtain control values and then the sequence of cold iodination, desulfation and radioiodination has to be applied. The data acquired may then be submitted to subtractive analysis thus reducing the background of radioiodination due to incomplete saturation of iodine binding sites.

3.4. Spiking of the same fraction with CCK4-S

For internal control of our reaction sequence (quantification and recovery), the fraction was spiked with 4 p*M* CCK4-S (2.2 μg/ml). Fig. 4C shows the sample plus CCK4-S after "cold" iodination, Sep-Pak extraction, desulfation with

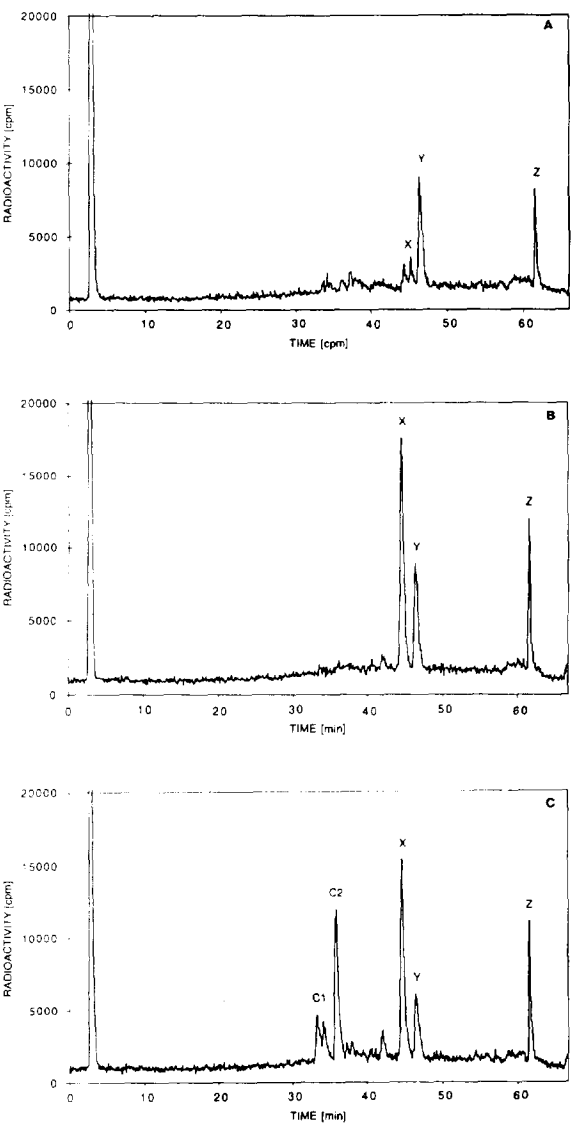

Fig. 4. RP-HPLC radiomonitored chromatograms of radioiodinated samples from a hemofiltrate fraction. (A) Radioiodination after first "cold" iodination and Sep-Pak extraction (X, Y, Z = detected peaks). (B) Radioiodination after first "cold" iodination, Sep-Pak extraction and desulfation for 3 min (X, Y, Z = detected peaks). (C) Radioiodination of the sample plus 4 p*M* CCK4-S (spiking of sample) first "cold" iodination, Sep-Pak extraction and desulfation (C1 = monoiodinated CCK4; C2 = biiodinated CCK4; X, Y, Z = detected peaks).

1 *M* HCl and radioiodination. Two further peaks appear in the chromatogram. Peak C1 is monoiodinated CCK4 (desulfated), peak C2 is biiodinated CCK4 (desulfated). Retention times and concentration are determined by standardization with different concentrations of CCK4-S using step III (data not shown). In the range 1–1000 p*M* a linear relationship with sufficient signal-to-noise ratio is achieved. Recovery of radioiodinated CCK4 in the crude hemofiltrate fraction is $>95\%$. Without desulfation no peaks eluting at the positions C1 and C2 were found in radioactive measurement, which is consistent with the absence of tyrosine in CCK4-S and the absence of other iodine binding sites (data not shown). This spiking experiment shows that quantitation of known sulfated peptides added to crude peptide fractions is possible using this method.

One observation from Fig. 4C has to be discussed in detail. The spiking of the crude peptide fraction with one sulfated peptide results in the generation of two radioactive products after completion of the entire reaction sequence. In earlier experiments the identity of C1 and C2 was shown to represent CCK4 (monoiodinated, C1 and biiodinated, C2). Again, complete biiodination is not consistently achieved within 120 s incubation time. The difference in retention time for small peptides (≤ 5 amino acids) is explained by the increased hydrophobicity in biiodinated products. Peptides with more amino acid residues show no significant difference in retention time for their mono- or biiodinated products. This problem with small peptides can be solved using cation- or anion-exchange chromatography. The hydrophobic iodines play no significant role in this case. Results can be obtained in the same quality (data not shown). In general, all chromatographic media are suitable.

4. Conclusions

The HPLC method of differential iodination is a helpful tool for the labeling and detection of sulfated peptides in biological samples. Even complex mixtures containing a high number of non-sulfated peptides can be screened for the presence of small amounts (low picomolar range) of tyrosine sulfate-containing peptides. In combination with amino acid analysis for control and quantification, the method of differential iodination using RP-HPLC coupled to a radioactivity monitor is fast and selective within the limits of 1–1000 p*M*. Our method allows the monitoring of metabolic processes involving sulfation and desulfation of peptides and proteins from biological sources.

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