HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBA-ANALOGUES OF OXYTOCIN; A METHOD FOR THE DETERMINATION OF SULFIDES AND SULFOXIDES IN PEPTIDES*

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Chromatographic properties of peptides derived from deamino-oxytocin, differing in the structure of the disulfide bridge region, were studied on a column with reversed phase. On the basis of chromatographic data the non-equivalence of the sulfur atoms in deamino-oxytocin was discussed. The analogues derived by oxidation or substutition of amino acids in the position 2 or 4 were also submitted to investigation. Rapid and selective oxidation of sulfide with periodate to sulfoxide, or the reduction of the latter with hydrogen bromide and acetone, was made use of for the determination of sulfide and sulfoxide in peptide containing sulfur-containing amino acid. The method was checked with free and protected peptides.

We have been studying the analogues of neurohypophysial hormones** with a modified disulfide bridge[2-10] for some time. In connection with biological and physico-chemical investigations of these analogues we decided to find out to what extent the modification of the disulfide bridge affects chromatographic properties of the analogue and whether it would be possible to make use of the data obtained in this way for correlation with the results of the above mentioned studies.

Generally it may be stated that a decrease in the polarity of a molecule increases the capacity factor of this compound (if columns with reversed phases are used), especially if the change does not affect the molecule's conformation. If the shape of the whole molecule is changed, for example in consequence of substitution of some amino acid for its optical antipode, or for a structural element requiring a change in spatial orientation (for example a sterically demanding amino acid, or on the

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** The nomenclature and the symbols used follow the proposals from literature[1]. The amino acids used were of L-configuration, unless stated otherwise. The abbreviation Thz means thiazolidine-4-carboxylic acid, Tpi means 4-thiapipecolinic acid (perhydro-1,4-thiazine-3-carboxylic acid), Tmb means 2,4,6-trimethylbenzyl group.

contrary, Pro, Gly, β-Ala), it is often hard to estimate, how the value k' will be affected, since the resulting effect is the sum of a number of factors, including the difference in the solvation of the molecule in solution and at the moment of the interaction with the stationary phase. Substitution of the sulfur atom in the disulfide bridge by a methylene group should not affect the conformation of the molecule of a hormone (with the exception of the carba-bridge), and therefore it should be possible to discuss the effect of the substitution of individual sulfur atoms on the affinity of the molecule toward the stationary phase.

When synthetizing sulfur-containing peptides a situation very often arises when we cannot be certain that the prepared peptide is indeed in the form of a sulfide. If an oxidation of the sulfur to sulfoxide takes place during the synthesis, the final steps could be complicated for example by the following facts; the protecting group on the sulfur atom of cysteine would not be cleaved by acidolysis[11], treatment with sodium in liquid ammonia would generate cysteinesulfinic acid[11] instead of cysteine, or a product of lower biological activity would be obtained[10]. For the detection of sulfoxide several methods are available (for example the analytical colour test[12] or infrared spectroscopy), which, however, give a positive response even when the product is merely contaminated with the sulfoxide. In addition to this several indirect methods exist which are based mostly on different behaviour of the sulfide and sulfoxide in chemical reactions (for example[13–15]). The only possibility of quantitative determination of sulfoxide is X-ray photoelectronic spectroscopy[16], which is very demanding with respect to the necessary equipment. We have tried to work out a method based on liquid chromatography which would permit the determination of sulfide and sulfoxide in mixtures of peptides.

EXPERIMENTAL

The peptides used — with the exception of cyclodipeptides[17] — were prepared in our laboratory. Their structures are given in Table I and III. For chromatography Separon SI C—18 was used as stationary phase (a spherical silica gel with bonded octadecyl chains), particle size 6 μm, packed in a column 15 cm long and 6 mm I.D. (Laboratorní přístroje, Prague). The column was provided with a septum injection port LCI-02 (Laboratorní přístroje, Prague), adapted for a stop flow operation. A Milton Roy Instrument MiniPump (Laboratory Data Control), model 386—74 was used. The peptide material in the eluate was detected by UV absorption measurement at 230 nm, using a spectral UV analyser UVM-4 (Development Laboratories, Prague). Methanol and acetonitrile were of analytical grade and water was redistilled. The buffer of pH 4·5 was prepared[18] by addition of triethylamine into a 1% solution of trifluoroacetic acid. The phosphate buffer[19] was prepared on adjustment of pH of a 0·02M phosphoric acid solution to pH 7 by addition of a sodium hydroxide solution. The optimal flow-rate through the column was determined experimentally and all separations were carried out at 2 ml/min flow-rate, when the operation pressure was between 15 and 26 MPa, dependent on the mobile phase.

Oxidation of Deamino-1-carba-oxytocin

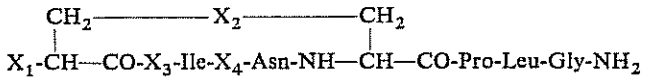
Deamino-1-carba-oxytocin (0·8 mg) was dissolved in methanol (300 µl) and the solution analysed chromatographically (Fig. 3*A*). A solution of sodium periodate (10 µl) of 50 mg/ml concentration (about 3 equivalents) was then added and the reaction mixture introduced into the column (Fig. 3*B*). The reaction was followed chromatographically for 2 h (Fig. 3*C* and 3*D*).

General Procedure in the Determination of the Oxidation Degree of Sulfur in the Peptide

First a solvent system should be selected in which the substance or mixture of substances under investigation shows a capacity factor about 2—3. Then an aqueous solution of sodium periodate is added to the sample (if only a small amount of the sample solution is available the operation can be carried out in a capillary, using a few microlitres) and the reaction mixture is introduced into the column immediately after mixing or after 10 minutes. If the chromatographic properties of the substance analysed do not change, 30 µl of acetone and 30 µl of hydrogen bromide solution in acetic acid (35%) are added to the sample of the substance (<0·1 mg) in a test tube with a ground glass stopper and the mixture is allowed to stand at room temperature for 5 min. The reaction mixture is evaporated to dryness at 20°C and reduced pressure (15 Pa) and the residue is codistilled twice with 50 µl of acetone. The residue is analysed chromatographically. When

TABLE I

The Structures of the Analogues Discussed



Compound	X_1	X_2	X_3	X_4	Ref.
Ia	NH_2	S—S	Tyr	Gln	29, 30
Ib	NH_2	CH_2—S	Tyr	Gln	2
Ic	H	S—S	Tyr	Gln	31
Id	H	CH_2—S	Tyr	Gln	4
Ie	H	S—CH_2	Tyr	Gln	5
If	H	CH_2—CH_2	Tyr	Gln	5
Ig	H	CH_2	Tyr	Gln	6
Ih	H	S	Tyr	Gln	6
Ii	H	CH_2—S—CH_2	Tyr	Gln	7
Ij	H	CH_2—S	Tyr	Glu	9
Ik	H	CH_2—S	Ile	Gln	8
Il	H	CH_2—S	Phe	Gln	8
Im	H	CH_2—S	Tyr(Me)	Gln	8
In	NH_2	CH_2—SO	Tyr	Gln	10
Io	H	CH_2—SO	Tyr	Gln	10
Ip	H	SO—CH_2	Tyr	Gln	10
Iq	H	CH_2—SO_2	Tyr	Gln	10

the analysed peptide contains acidolytically cleavable protecting groups a parallel experiment should be carried out in which acetone is substituted by acetic acid, and the material obtained in this manner compared with the product obtained on reduction, or if necessary the labile protecting groups eliminated before reduction.

RESULTS AND DISCUSSION

For the separation of our analogues triethylammonium trifluoroacetate buffer of pH 4·5 proved suitable. It is the buffer used by Belgian authors in combination with acetonitrile[18]. However, in view of the fact that for the achievement of equal retention times on a column with Separon SI C–18 as on a column with Bondapak

TABLE II

Values of k' of the Analogues of Oxytocin for Various Mobile Phases

Composition of the Mobile Phase: 1 buffer of pH 4·5–methanol (1 : 1), 2 buffer of pH 4·5–acetonitrile (4 : 3); 3 buffer of pH 7–water–methanol (3 : 3 : 4); 4 water–methanol (1 : 1).

Compound	Mobile phase			
	1	2	3	4
Ia	2·00	5·86	8·86	[a]
Ib	1·83			[a]
Ic	3·22			3·59
Id	2·93			3·21
Ie	2·00 (13·6)[b]	10·00	8·79	2·57
If	2·00 (13·3)[b]	9·60	8·35	2·57
Ig	1·62			1·97
Ih	2·54	11·4	11·4	2·95
Ii	2·57	12·8	12·2	2·95
Ij	2·33		4·02	1·00
Ik	6·43			
Il	8·72			
Im	8·42			8·43
In	1·56 and 1·60			[a]
Io	1·93 and 2·22 (6·06 and 7·50)[c]		8·22	2·07 (6·92 and 7·49)[d]
Ip	1·55			1·86
Iq	4·72			

[a] Cannot be determined in non-buffered system; [b] ratio of buffer pH 4·5 to methanol was 3 : 2; [c] ratio of buffer pH 4·5 to methanol was 11 : 9; [d] ratio water–methanol (3 : 2) .

C-18 or Lichrosorb RP-18 a much larger amount of acetonitrile is necessary in the mixture with the buffer (indicating a higher proportion of the fixed non-polar component on the silica gel Separon), we used methanol as the organic solvent.

As is evident from Table II we succeeded in the separation of almost all analogues differing merely in the structure in the region of the disulfidic bridge even when using mobile phases giving capacity factor values in the 1·7–3·5 range. In this phase only deamino-6-carba-oxytocin (*Ie*) and deamino-dicarba-oxytocin (*If*) would not separate, and the separation of the analogue with a decreased (*Ih*) and an increased (*Ii*) cycle was observable as a shoulder only. The dicarba (*If*) and 6-carba (*Ie*) compounds showed a hint of separation only when the increase in the content of the buffer in the mobile phase produced a capacity factor of about 13 (the separation factor value $\alpha = 1{\cdot}025$). When acetonitrile was substituted for methanol a slightly better separation of this pair was obtained ($\alpha = 1{\cdot}04$), and the analogues with a decreased and an increased ring (*Ih* and *Ii*) separated very well ($\alpha = 1{\cdot}12$). The use of the

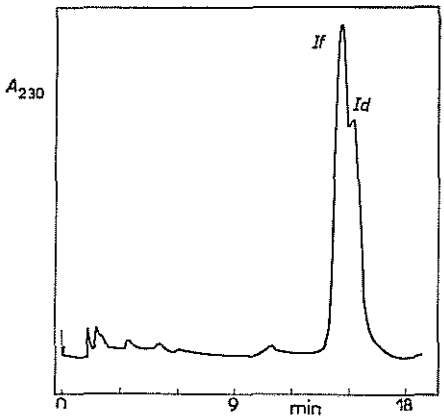


FIG. 1

Chromatogram of a Mixture of Deamino--dicarba-oxytocin (*If*) and Deamino-6-carba--oxytocin (*Ie*)

Mobile phase: buffer pH 7–water–methanol (3 : 3 : 4).

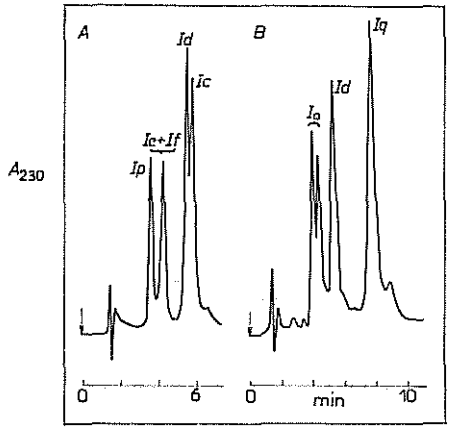


FIG. 2

Chromatography of a Mixture of Sulfoxide of Deamino-6-carba-oxytocin (*Ip*), Deamino--dicarba-oxytocin (*If*), Deamino-6-carba-oxytocin (*Ie*), Deamino-1-carba-oxytocin (*Id*), Deamino-oxytocin (*Ic*), Sulfoxide of Deamino-1-carba-oxytocin (*Io*) and Sulfone of Deamino-1-carba-oxytocin (*Iq*)

Mobile phase: buffer of pH 4·5–methanol (1 : 1).

phosphate buffer of pH 7 in combination with methanol led to a slightly better separation of 6-carba (*Ie*) and dicarba (*If*) analogues (Fig. 1), while the substances with a different cycle (*Ih* and *Ii*) separated less well ($\alpha = 1{\cdot}07$).

The substances, differing merely in the fact that one of them contained a sulfur atom in the bridge and the other contained a methylene group instead, were eluted in the order CH_2-compound, S-compound (compare the k' of compounds *Ib* and *Ia*, *Id* or *Ie* and *Ic*, *If* and *Id* or *Ie*, *Ig* and *Ih*; the couple of the cyclodipeptides (c(Pro-D-Phe) and c(Thz-D-Phe) – k' 1·71 and 2·71) behaves in the same manner. The substitution of sulfur with a methylene group evidently decreases the affinity of the molecule to the non-polar stationary phase. Since exactly the opposite behaviour would be easily explicable by a decrease of the polarity of the whole group investigated, the explanation of this proved fact should be sought in the interaction with the stationary phase where the peptide molecule occurs in solvated state. When the sulfur of the disulfidic bridge is exchanged for a methylene group the coordination with the solvent molecules (for example with water) can be deteriorated, and the polarity of the whole complex increases. Among the group of substances containing the S—S, CH_2—S, S—CH_2 and CH_2—CH_2 grouping in the bridge the substances with a reverse direction of the CH_2—S bond (Fig. 2*A*) differ substantially in their chromatographic behaviour. From Table II it is evident that the substitution of S—S for CH_2—S brings about a relatively small change in the retention time of the substance formed ($\alpha = 1{\cdot}1$), while the exchange of S—S for S—CH_2 has a much more pronounced effect ($\alpha = 1{\cdot}61$). When passing from the S-CH_2 grouping to the CH_2—CH_2 grouping the change of the capacity factor is again very small ($\alpha = 1{\cdot}02$). The difference between the retention times of substances containing the CH_2—S and CH_2—CH_2 grouping is evidently much larger ($\alpha = 1{\cdot}63$). Hence, it is again evident that both sulfur atoms are quite unequivalent, and it seems that the sulfur atom in the position 1 (*i.e.* in carba 6 analogues) is in some way hidden inside the whole molecule, so that its effect on the interaction with the environment is much smaller than the effect of the sulfur atom in the position 6. This is in agreement with the finding[10] that oxidation of the sulfur in the position 1 affects the biological activities of the carba analogues much less than the oxidation of the sulfur in the position 6. In analogues that are eluted later (*Ic* and *Id*) an interaction between the free electron pairs of the sulfur atom and the aromatic ring of tyrosine is possible according to the CD measurements[20], while in compounds with a lower k' value (*Ie* and *If*) this interaction is improbable. (In connection with this it may be mentioned that of the couples of substances c(Thz-Phe) and c(Thz-D-Phe) or c(Tpi-Phe) and c(Tpi-D-Phe) those are also eluted later in which it could be shown by CD spectra[21] that an interaction exists between the sulfur atom and the aromate, even though a *cis*-substitution of the six-membered ring exists in the first case and a *trans*-substitution in the second – see Table III.) This interaction may also be responsible for the different chromatographic behaviour of the given compounds. If it does occur, the aromatic residue is fixed in a certain

position, while if not, the residue has a much higher degree of freedom, which can lend the molecule a certain degree of polarity.

Chromatographic behaviour of sulfoxides *Io* and *Ip* also corresponds to the idea of non-equivalence of the two sulfur atoms. The separation coefficient of the given sulfoxide and corresponding sulfide is smaller in deamino-6-carba-oxytocin (*Ie* and *Ip*, $\alpha = 1{\cdot}29$) than in deamino-1-carba-oxytocin (*Id* and *Io*, $\alpha = 1{\cdot}32$ or $1{\cdot}52$, respectively). See Figs 2*A* and 2*B*. In the case of 1-carba compound (*Io*) a separation of diastereoisomers formed by the creation of a further asymmetry centre on the sulfur also takes place, while in the case of 6-carba compound (*Ip*) the peak of the sulfoxide could not be separated, which supports the idea of greater accessibility to the sulfur atom in the position 6; in the case when sulfur is in the position 1 (in carba-6-oxytocin) sterical reasons may force the formation of a single diastereosiomer. Another explanation of this fact stems from the idea of the interaction of the sulfur atom with the aromatic residue, which is disturbed in a different way in both diastereoisomers of deamino-1-carba-oxytocin sulfoxide (*Io*). The diastereoisomers of the sulfoxide of 6-carba analogue need not differ in their effect on the conformation of the rest of the molecule, and therefore their separation may not be achieved.* When a buffer of pH 7 is used, a separation of stereoisomers does not take place and when a mixture of methanol and water is used the separation is much less clear (Table II). The percentual representation of optical isomers in the sulfoxide of deamino-1-carba-oxytocin (*Io*) is dependent on the method of preparation of this compound. The chromatographic picture of the substance prepared by stepwise construction of the chain with the sulfoxide of amino acid (Fig. 2*B*) is different from

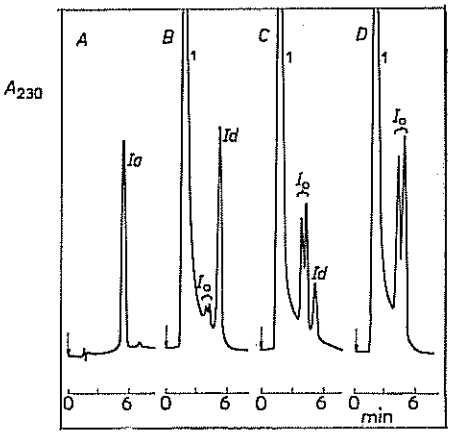


FIG. 3

Chromatographic Observation of the Oxidation of Deamino-1-carba-oxytocin (*Id*) with Sodium Periodate (*I*) under Formation of a Mixture of Stereoisomers of Corresponding Sulfoxide (*Io*). Mobile Phase: Buffer of pH 4·5–Methanol (1 : 1)

A Starting solution of deamino-1-carba-oxytocin, *B* reaction mixture immediately after addition of sodium periodate, *C* reaction mixture after 10 min, *D* reaction mixture after 120 min.

* *Note added in proof:* According to the new findings (Lebl M., Barth T., Jošt K.: Proc. XVI th Europ. Pept. Symp., Helsingor 1980; in press) this explanation looks more probable.

the record obtained during the oxidation of deamino-1-carba-oxytocin (*Id*) with periodate, followed chromatographically (Fig. 3*A* – *D*). This situation may be caused by different accessibility of the sulfur atom for the oxidant in the amino acid derivative and in a cyclic peptide with a relatively fixed conformation. However both mixtures of diastereoisomers display identical activity[10] within the limits of the errors of biological tests. The separation of the diastereoisomers of sulfoxides also does not take place in the case of compounds *Ih* and *Ii* (if the diastereoisomers are formed at all), and in the case of substance *In* the separation is indistinct, while in the case of sulfoxides of the analogues derived from deamino-1-carba-oxytocin *Ij* and especially *Ik* and *Im* the diastereoisomers differ considerably (Table III).

The higher oxidation degree of this type of substances is sulfone which, when derived from deamino-1-carba-oxytocin has a relatively surprising chromatographic behaviour (Fig. 2*B*). In view of the expected higher polarity it should possess a lower

TABLE III

Values of k' of Peptides Containing Sulfur and of Corresponding Sulfoxides

Compound	$k_{S'}$	$k_{SO'}$	Ratio of buffer pH 4·5 to methanol
Ib	1·83	1·56 and 1·60	1 : 1
Id	2·93	1·93 and 2·22	1 : 1
Ie	2·00	1·55	1 : 1
Ih	2·54	1·72	1 : 1
Ii	2·57	1·32	1 : 1
Ij	2·33	1·53 and 1·75	1 : 1
Ik	1·78	1·07 and 1·63	2 : 3
Im	2·15	1·36 and 2·00	2 : 3
Met-Leu-Gly	1·86	0·57	3 : 1[a]
Ile-Gln-Asn-Hcy(C_2H_4COOH)-Pro-Leu-Gly-NH_2	1·36	0·79	2 : 1[a]
Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH_2	5·71	3·28	2 : 3
Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH_2	2·57	1·64	2 : 3
Z-Cys(Bzl)-Gly-OEt	2·79	1·07 and 1·21	3 : 7
Z-Cys(Bzl)-Gly-Gly-OEt	2·15	0·83 and 0·90	3 : 7
Z-Cys(Tmb)-Pro-Leu-Gly-NH_2	2·86	1·28 and 1·43	1 : 4
c(Thz-Phe)	3·29	0·86	1 : 1
c(Thz-D-Phe)	2·71	0·64	1 : 1
c(Tpi-Phe)	3·43	0·71	1 : 1
c(Tpi-D-Phe)	4·21	0·86	1 : 1

[a] Buffer of pH 7.

value of the capacity factor than the starting sulfide. A possible explanation for the increased value of k' is that by this oxidative change the conformation of the molecule of the analogue is completely changed and that the interaction with the stationary phase is also dramatically changed.

Further studied analogues are derived from deamino-1-carba-oxytocin and they differ by some amino acid in the peptide chain. When glutamic acid is substituted for glutamine an increase in the polarity of the molecule takes place (to which a decreased k' value corresponds), which becomes distinctly evident when a mobile phase with higher pH value is used (phosphate buffer of pH 7), or when a non-buffered system with pure water is used (Table II). When the hydroxyl group of tyrosine in the position 2 is eliminated (by introduction of phenylalanine) or substituted by a methoxy group (in the analogue containing O-methyltyrosine) an approximately equal increase in the capacity factor value is observed, which is even higher than in the substitution of tyrosine by leucine.

Oxidation with sodium periodate can be made use of to determine whether the substance prepared is in the form of a sulfide or sulfoxide, or which of the chromatographic peaks corresponds to the substance containing the sulfide or the sulfoxide group. After addition of periodate to the solution of the analogue tested in the form of sulfide, the formation of a substance with a lower capacity factor value may be observed within minutes, *i.e.* of a sulfoxide. If the chromatographic properties of the substance investigated do not change, it may be stated with a rather high probability, that the substance prepared is a sulfoxide, especially if a similar study is carried out in several solvent systems. In order to confirm this assumption a reduction of the substances studied or a mixture of substances can be carried out. Reduction with thiols (for example mercaptoethanol[22]) is less suitable for this purpose, and because it requires a considerable excess of the reagent, which then interferes in the chromatographic evaluation of the reaction. The reaction with hydrogen bromide and acetone represents a substantially faster reduction[10,22], taking place under milder conditions with a quantitative yield and affording no non-volatile by-products (see Experimental). When it is used for substances with acidolytically cleavable groups it is also necessary to carry out a similar reaction with hydrogen bromide in the absence of acetone, or to eliminate the protecting groups before reduction. The *o*-nitrobenzenesulfenyl protecting group should be also eliminated before reaction with periodate, because it is split off[11] with this reagent. If the chromatographic properties of the substance analysed do not change even after reduction, we may be faced with a sulfone the formation of which, however, is in most instances not very probable and which can be detected by other methods easily (for example by amino acid analysis) or with a substance that does not contain sulfur. Sulfur in the form of a sulfonium salt can be detected chromatographically on the basis of its thermal lability, when it is converted on heating to sulfide[23,24] (in the case of tert-butyl-sulfonium salt), or also by NMR spectrometry[24,25].

The values of the capacity factors of various peptides, containing sulfur and corresponding sulfoxides, on which this method was tested, are given in Table III. As is evident, we had not only carba-analogues of oxytocin, but also free and protected peptides of various types, or even cyclopeptides for which it applies that the corresponding sulfoxide differs chromatographically and that it is as a rule eluted earlier. A similar behaviour of sulfoxides was also described, for example, for the fragments[26] and analogues[27] of ACTH or calcitonin[28], so that it may be considered as rather general. When it is already determined which of the chromatographic peaks belongs to the sulfide and which to the sulfoxide a quantitative evaluation of their content may also be carried out. The error originating from the ignorance of the extinction coefficients of pure compounds decreases with increasing size of the peptide (if the measurement is done in the region of the absorption of amide bonds), or it is completely eliminated when the extinction is recorded in the region of the absorption of aromatic rings.

In conclusion it may be said that the technique of high-pressure liquid chromatography on reverse phase permits the differentiation of such structural details in substances with a molecular weight of about 1000 as arise for example, from the substitution of sulfur by a methylene group, or of a CH_2—S group by a S—CH_2 group, or also from the spatial orientation of the oxygen atom of the sulfoxide group. It also seems that the chromatographic data can be correlated to a certain extent with the conformation of the peptide, which has been demonstrated by the analysis of the non-equivalence of the sulfur atoms in the disulfide bridge of oxytocin. The differentiation of the sulfide and the sulfoxide by means of chromatography connected with a rapid and selective oxidation of sulfur with sodium periodate or the reduction of the sulfoxide with hydrogen bromide in acetone, may be used for the determination of these oxidation degrees of sulfur in newly prepared peptides.

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