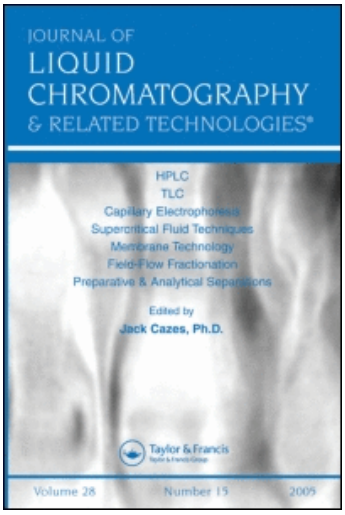

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SOME ASPECTS OF SIMULTANEOUS DETERMINATION OF PYRIDINOLINES AND DESMOSINES AS COLLAGEN AND ELASTIN RESORPTION MARKERS BY MEANS OF REVERSE PHASE HPLC, UTILIZING FLUORESCENCE AND/OR UV-ABSORPTION MONITORING

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SOME ASPECTS OF SIMULTANEOUS DETERMINATION OF PYRIDINOLINES AND DESMOSINES AS COLLAGEN AND ELASTIN RESORPTION MARKERS BY MEANS OF REVERSE PHASE HPLC, UTILIZING FLUORESCENCE AND/OR UV-ABSORPTION MONITORING

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ABSTRACT

Isocratic reverse phase HPLC method was elaborated and optimized for simultaneous determination of pyridinoline (PD), deoxypyridinoline (DPD) and isodesmosine (IDES) in urine and in tissues within approximately 16 minutes. Mobile phase is 0.02 M heptafluorobutyric acid (HFBA) with 0.01 M $(NH_2)_2SO_4$ and 8 - 12 % acetonitrile (ACN), at pH 1.55, flow rate 0.5 mL/min. and temperature of 40°C. The addition of ammonium sulphate affects positively the quality of chromatographic separation. The glass column 150 x 3 mm is filled with 7 μm spherical silica C_{18}. The concentrations of determined substances are monitored by detector of fluorescence at $\lambda_{EX}/\lambda_{EM}$ = 297/400 nm for both PD and DPD, and at $\lambda_{EX}/\lambda_{EM}$ = 275/320 nm for IDES. Both PD and DPD are measurable with the same sensitivity limit of 200 femtomols; in the case of IDES the determination sensitivity is approximately 5x lower (1 pmol).

Since IDES concentration in urine is considerably lower, it means that approximately 25 mL of the sample must be processed and the time of preparatory work is, therefore, considerably increased. In this system, desmosine (DES) is practically not detectable by fluorescence. It was verified by comparison of detection sensitivity in fluorescence and UV-absorption measurements that fluorescence is substantially more sensitive (with the detectors used by us) for the determination of PD, DPD, and IDES. If it is necessary to quantify DES as well, UV-absorption must be measured, and therefore the urine must be concentrated at least 5 times more. HPLC separation itself is very effective, on baseline, and with high number of theoretical plates of the column for all relevant substances. HPLC assays of tissue samples are of higher quality and more transparent than those of urine.

INTRODUCTION

Hydroxylysylpyridinoline (pyridinoline, PD) and lysylpyridinoline (deoxypyridinoline, DPD) are stable, non-reducible, crosslinking elements, which occur only in mature, fibrous collagen, located on site. These substances are created by the sequence of both enzymatic and chemical reactions, in which lysinohydroxylase participates at intracellular level, and lysinooxidase at extracellular level, followed by aldol condensation, rearrangements, oxidation, and dehydration, and during these processes PD is created out of 3 hydroxylysyl residues and DPD out of 2 hydroxylysyl and 1 lysyl residues.[1,2]

Desmosine (DES) and its isomer isodesmosine (IDES) are created analogously in mature elastin out of the precursor tropoelastin through the series of similar reactions out of 4 lysyl residues.[3] During the metabolism of the connective tissues, PD, DPD, DES, and IDES are released either as free (in the case of PD and DPD approximately 40%)[4] or peptide-bound compounds into the blood circulation. At the very end in the case of normal kidney function they are concentrated in urine, where the determination of their concentrations serves as specific and sensitive indicator of collagen and elastin resorption kinetics. In the case of collagen the levels of PD and DPD in urine (UPD or UDPD) are more specific markers of osteo-resorption than formerly more usual measurements of hydroxyproline.[5]

Besides the ligamentum nuchae, larger concentration of elastin occur in elastic cartilages, large blood vessels, lung, skin, and also in liver and kidneys. The structure of elastin provides, at one side, the features of elastomer and on the other side, the crosslinks of desmosine and isodesmosine ensure considerable strength. The concentration of all 4 markers in urine is not

influenced by the diet,[6] they are not metabolized in liver, and they do not occur in other proteins.[7,8] The determination of local DES and IDES concentration is sometimes also used for the calculation of elastin content in tissue.[9] The increased concentration in urine serves also as a good indicator of some pathological processes in soft tissues, for instance in the case of lung diseases[10] (obstructive lung disease, cystic fibrosis, emphysema), skin diseases[11] (local scleroderma), diseases of blood vessels and internal organs (e.g. in the case of systemic sclerosis).[12]

In the case of affection of connective tissue, where collagen and elastin are present together in many organs, the combination of excretion values of crosslinks of both proteins, or in the case of PD and DPD also, the ratio of their values itself can provide very useful information. For instance in the case of one type of Ehlers-Danlos syndrome, DPD/PD ratio is approximately 6 in comparison to the usual value of approximately 0.2.[13] In systemic sclerosis both DES and IDES increase approximately 2-3 times, PD and DPD approximately 3-4 times;[12] in the period of 2-5 weeks after the parturition when uterus is being contracted DES values are approximately 5 times higher and PD values approximately 4-21 times higher than in controls,[14] etc. DES and IDES correlate with each other very well, and their ratio is usually ≈ 1.[15,16]

The crosslinking elements of collagen are detected usually by ELISA methods, which are used especially for screenings in large groups of patients.[17] Furthermore, there are many methodological alternatives for reverse phase HPLC[18,19] under slightly acid pH, during which the strongly polar pyridinium crosslinks are converted into relatively nonpolar components by ion-pairing substance, e.g. heptafluorobutyric acid (HFBA) or alkyl sulphate. It appeared that the detected values are frequently overestimated by ELISA, which is explained by a possibility of nonselective reactions with other pyridinium components or with peptide-bound crosslinks of different sizes.[20,21]

There is a similar situation for the determination of DES and IDES, where (usually older) works based on RIA and ELISA[22,23] showed relatively high values of seemingly positive background, indicating presence of secondary nonselective reactions.[15] Chromatography is considered as the only reliable analytical method, apparently because it physically separates all relevant substances and demonstrates them on the record, and it is used frequently as a method of reference. In the United States, considerable attention is devoted recently to the possibility of simultaneous determination of PD, DPD, DES, and IDES (the group of Stone, Franzblau et al.) based on the principle of reverse phase HPLC in the presence of ammonium chloride with octyl sulphate. With this method the concentration of separated substances is determined by UV-absorption and from the value of decreased specific radioactivity of their isotopes.

In the Czech Republic we have introduced a HPLC method, which is used for the determination of PD and DPD in urine and serum[24,25] based on the principle of separation on strong cation exchanger, and the detection is carried out by monitoring of fluorescence.

The aim of this work is to develop a modified reverse phase HPLC for the simultaneous determination of PD, DPD, DES, and IDES with the presence of heptafluorobutyric acid and with the utilization of fluorescence detection (both DES and IDES also have their natural fluorescence).[26] The process of separation has to be optimized; the method has to be calibrated and applied for several randomly selected samples of urine and tissues. A mutual comparison of detection sensitivity by fluorescence and UV-absorption will be carried out as well.

Expected Problems

a) During simultaneous determination of all these markers it will be necessary to match, in a suitable way, both the requirement of fine separation within both pairs and the concurrent need of not so much fine separation between both pairs.

b) Since we do not have available a fluorescence detector with a possibility to change excitation (λ_{EX}) and emission (λ_{EM}) wavelengths, it will be necessary to work in such a way that there will be enough time for manual manipulation with the detector and the integrator.

c) Due to the fact that in the literature the levels of DES and IDES in urine are reported to be more than 10 times lower than the level of PD,[3,28] and the quantitative values of the intensity of their fluorescence response are not known, there may be a necessity to decrease the level of sensitivity of PD detection in such a way that its signal would not exceed the measurable scope of values of the instrument, and at the same time it may be necessary to process a relatively voluminous urine sample, which will make the method rather demanding for materials and time.

EXPERIMENTAL

Urine samples were collected from the patients of the Institute of Rheumatology in Prague, with their consent. In the patients the levels of UPD and UDPD were examined for diagnostic and therapeutic purposes. PD and DPD standards were prepared in our own laboratory, DES and IDES standards were purchased at ICN (Costa Mesa, CA, USA). Heptafluorobutyric acid (HFBA), acetonitrile (ACN), elastin from bovine ligament, and spherical

cellulose CC31 were purchased at Sigma-Aldrich in Prague, Czech Republic. Sodium sulphate norm., ammonium sulphate norm., sodium hydroxide, acetic acid, n-butanol, and hydrochloric acid, all in p.a. quality, were purchased at Penta, Chrudim, Czech Republic. Demineralized water is produced directly in our institute.

PD and DPD Standards

1,000 mL of urine, obtained by pooling from children aged 7 ± 5 years (i.e. mean ± S.D.), which according to previous HPLC analysis contained 1.336 μg PD/mL and 0.298 μg DPD/mL, were freeze-dried in two containers of 500 mL. The evaporation residue was dissolved in 250 mL of 6 M HCl, and was divided into 50 vials of 5 mL each. After washing with gaseous N_2 the samples were hydrolyzed at 110°C for 16 hours. The content of each vial was mixed with 5 mL of acetic acid and 20 mL of n-butanol, mixed, and gradually transferred into a PE column (20 mL) filled with sedimented spherical cellulose CC31, which bed was protected both above and below with porous PP frit, and previously conditioned with the mixture of n-butanol : water : acetic acid (4:1:1 by volume). The columns were washed with 30 mL of the mixture n-butanol - water - acetic acid (4:1:1 by volume) using manual vacuum extractor Supelco (Sigma-Aldrich, Prague, Czech Republic). Irreversibly, absorbed collagen and desmosine crosslinking elements were desorbed with 6 mL of water, evaporated in rotary vacuum evaporator SpeedVac (Philadelphia, PA, USA), and reconstituted in 5 mL of 0.01 M HFBA. Aliquots of 0.5 mL each were injected into semipreparative HPLC column (SEPARON SGX C18, 250x10 mm, Tessek, Prague, Czech Republic). Mobile phase was 0.01M HFBA, at pH 1.86, with 24% ACN, at flow rate 2 mL/min., and temperature 40°C.

Fluorescence was detected at $\lambda_{EX}/\lambda_{EM}$ = 297/400 nm and the fractions, which correspond to PD and DPD, were pooled and freeze-dried in containers of 500 mL. The evaporation residues were repeatedly dissolved and dried, until only one has remained of each crosslink. Gravimetric finding was 0.89 mg PD (i.e. approximately 66.6 % of the quantity anticipated according to the previous HPLC analysis) and 0.18 mg DPD (i.e. approximately 59.7 %). Relatively large losses of both standards are probably due to the lengthy procedure and inaccurate weighing of very small quantities.

Urine Samples

Twenty-five mL of urine were mixed with the same volume of concentrated HCl and hydrolyzed in the atmosphere of N_2 at 100°C for 16 hours. The product of hydrolysis was mixed with 50 mL of acetic acid and 200 mL of

n-butanol. The mixture was processed (analogously as described above) in 10 aliquots on columns (PE, 20 mL), filled with 2 mL of sedimented spherical cellulose CC31, which was previously conditioned in the mixture of water - acetic acid - n-butanol in the ratio of 1:1:4 (by volume). The columns were washed with 30 mL of the mixture of water - acetic acid - n-butanol (1:1:4 by volume), crosslinks were desorbed two times with 4 mL of water and dried in the rotary vacuum evaporator. The evaporation residues were dissolved in 200 μL of 0.5 M CH_3COOH, individual fractions were put together, again dried, and the evaporation residue was dissolved in 200 μL of 1% HFBA solution with 0.01 M $(NH_4)_2SO_4$. The original urine samples were concentrated 125x, in such a way and the volume of 20 μL was used for injection into HPLC analytical column.

Tissue Samples

Tissue samples from aorta and lungs were isolated from female rats of Lewis strain LEW/Crl/CrlBR bred by Charles River Deutschland. Samples of aorta of 150 mg wet weight, and of lung of 1,500 mg wet weight were taken for this work. Remainders of undesired tissues were removed from the samples, which were then frozen to -20°C and immediately freeze-dried in rotary vacuum evaporator SpeedVac for 24 hours. Dry tissues were cut into slices approximately 1 mm thick, extracted in 6 parts of chloroform/methanol mixture (2:1 by volume) for the period of totally 48 hours, again dried in rotary vacuum evaporator, and weighed. The weight of dry aorta sample was 35 mg (23.3 % of dry matter in the original sample) and in the sample of dry lung it was 215 mg (14.3 % of dry matter in the original sample). Dried tissues were mechanically crushed, and 35 mg of aorta (marked A) and 200 mg of lung (marked L) were dispersed in 2 mL of 6 M HCl and hydrolyzed under N_2 atmosphere at 110°C for 16 hours.

The products of hydrolysis were filtered and 1 mL of each was mixed with 1 mL of acetic acid and 4 mL of n-butanol. Each sample of the volume of 6 mL was then processed in a standard way in 3 columns filled with spherical cellulose (i.e. 2 mL sample per column), washed with 20 mL of the mixture of n-butanol - water - acetic acid (4:1:1 by volume): crosslinks were desorbed from cellulose with 3 mL of water and evaporated. The evaporation residue was twice dissolved in 200 μL of 0.5 M acetic acid, and the fractions containing crosslinks from the same type of tissue of the volume of 1.2 mL (i.e. 3 evaporation residues of 2 x 200 μL each) were again evaporated and dissolved in 1 mL of 1% HFBA with 0.01 M ammonium sulphate. The volume of 20 μL was used for injection into an HPLC column.

Elastin Hydrolysate

Totally 5 mg of elastin from bovine ligament were hydrolyzed in 2 mL of 6 M HCl (16 hours, 110°C). 2 mL of acetic acid and 8 mL of n-butanol were added to the product of hydrolysis. The mixture was processed in 10 aliquots on CC31 cellulose, and after purifying and concentrating in a similar way as in the case of urinary hydrolysate, the evaporation residues were reconstituted in 200 μL of 1% HFBA with 0.01 M $(NH_4)_2SO_4$.

High Performance Liquid Chromatography

The instrument used was a liquid chromatograph of Spectra-Physics (San Jose, CA, USA), type SP8100 with autosampler SP8110 and Chrom-Jet integrator SP4400 of the same manufacturer. Fluorescence was monitored with fluorescence detector RF 535 (SHIMADZU, Kyoto, Japan), and occasionally with UV/VIS detector SP 8440 (Spectra-Physics). For the mobile phase 0.02 M HFBA was used as ion-pairing agent with 0.01 M $(NH_4)_2SO_4$ and 8 - 12% acetonitrile as organic modifier. Glass column of CGC type, 150 x 3 mm, filled with spherical silica gel of particle size 7 μm, with surface modified octadecylsilane (Separon SGX C18, Tessek, Prague, Czech Republic) was thermostated to 40°C (higher temperature accelerates concentration equilibrium on sorbent surface, and in such a way increases the number of theoretical plates of the column). The flow rate of the mobile phase was 0.5 mL/min.

RESULTS AND DISCUSSION

Both PD and DPD are usually detected with fluorescence monitor at the excitation wavelength of 297 nm and emission wavelength of 400 nm, and this setting was also used in this case. However, for DES and IDES the absorption maxima are reported at the wavelengths of 275 and 280 nm,[26] which should be therefore used as excitation values for fluorescence detection. It is necessary to optimize the separation conditions in order to acquire the following:

1) There must be time lag enough between the pyridinolines and the desmosines to allow manual setting of the fluorescence detector and the integrator parameters.
2) Separation of PD, DPD and at least of one desmosine should be on the baseline.
3) The peaks should be symmetric, as narrow as possible, and therefore also as high as possible.
4) The analysis time should be as short as possible (isocratic separation).

It is known, from literature sources, that the analysis is mostly performed at the presence of proper concentration of ACN and usually with 0.01 M HFBA. Higher concentration of HFBA and, therefore, lower pH have positive influence on increased retention of substances. However, this system had low efficacy in our case; the peaks were asymmetric, and the resolution coefficient R_S (defined as the values of distance of both peak maxima divided by the average value of peak basal width) was low. Sometimes pH of the system is increased by titration with sodium hydroxide. However, in our case this raised decrease of the efficacy, supported tailing and, furthermore, it strongly reduced retention and thus also total separation quality. The following decrease of acetonitrile concentration in mobile phase negatively affected the accuracy of its dosage and thus also the reproducibility of retention times.

Therefore, the mobile phase with different molality of HFBA (0.01-0.03 M) was modified with addition of salts. The organic modifier was 40% aqueous solution of ACN. The influence of different concentrations of salts (NH_4Cl, Na_2SO_4, $(NH_4)_2SO_4$), which restrict the influence of nonspecific interactions of separated substances with, for instance, some residual silanol groups of sorbent, was assayed. These salts contribute, on one hand, to higher number of theoretical plates of the column, on the other hand, however, they decrease retention times of separated substances. Ammonium chloride, which had been used at first, was replaced by sodium sulphate, due to the fact that negative influence of Cl^- ions on the magnitude of fluorescence signal was reported.[27] The latter substance, however, decreased retention and generally impaired separation efficacy. Therefore it was again replaced, namely by ammonium sulphate, which decreased pH and increased retention, and in such a way best results were achieved.

Based on the series of HPLC experiments (table not shown), the solution of 0.02 M HFBA with addition of 0.01 M ammonium sulphate was selected as a part of the mobile phase. The quantity of ACN was modified in such a way (usually between 8 % and 12 %) that the separation of PD and DPD is optimum and the time delay before the elution of desmosines allows already mentioned manual setting of the integrator and fluorescent detector. Under such conditions, the resolution coefficient R_S for PD and DPD equaled 2.1. For a good separation it is required that $R_S \geq 1$. The number of theoretical plates of the column of 150 mm of length (calculated from retention times and the widths of peaks in one half of their size) was ≈ 6000, which is a very good result.

On Fig.1 there is a demonstration of two HPLC analyses, where IDES standard and elastin hydrolysate, respectively, were added to urine samples with both pyridinolines. During the time interval designated ΔT, it is necessary to change manually the setting of $297_{EX}/400_{EM}$ nm for the determination of PD and DPD, to the optimum of $275_{EX}/320_{EM}$ nm for IDES, and furthermore to

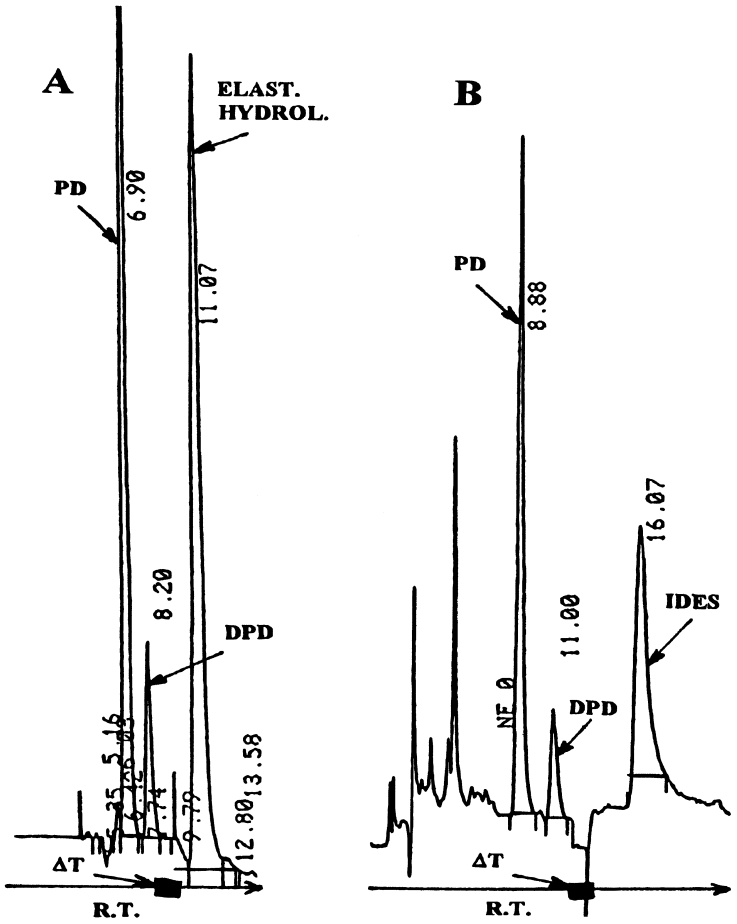

Figure 1. Record of HPLC analysis of urine samples spiced with: A - elastin hydrolysate, B - isodesmosine standard. The quantity of added ACN: A - 10%, B - 8%. ΔT - time interval for the change of parameters of integrator and fluorescence detector.

equalize baseline shift and to engage higher level of noise filtration (all in fluorescence detector). At the same time it is advisable to switch off integration temporarily, to decrease sensitivity and to reset existing signal (all in the integrator).

For the purpose of quantitative determination of all 4 crosslinking elements, the calibration solutions of isolated standards were prepared, and their fluorescence response (integrated peak area) was measured. The results of the calibration are on Fig. 2.

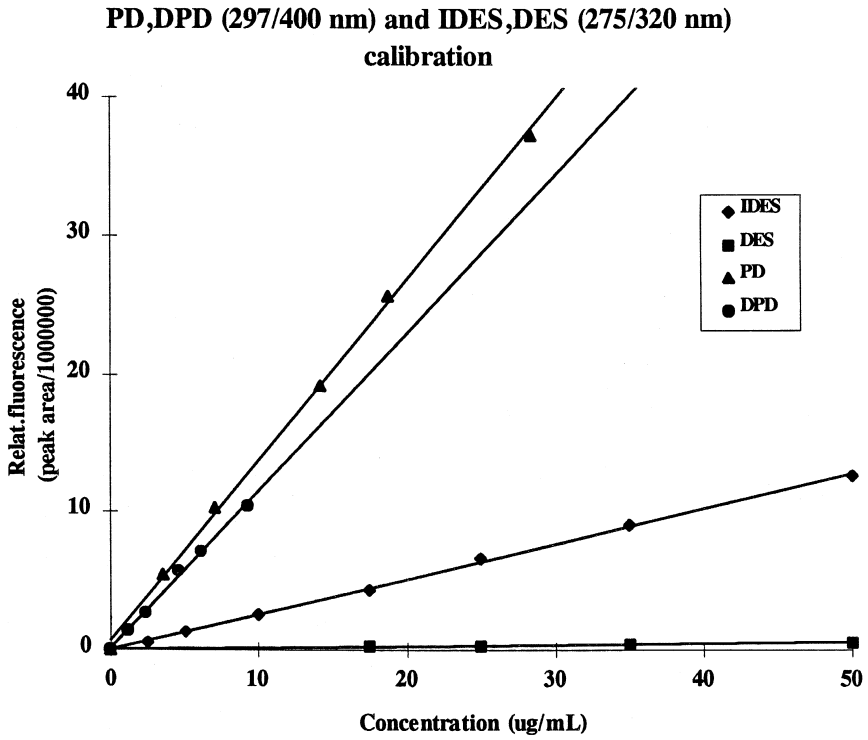

Figure 2. Fluorescence calibration curves for PD, DPD, IDES and DES, expressed as the value of integrated peak area related to the concentrations. The measurement was taken at $\lambda_{EX}/\lambda_{EM}$ = 297/400 nm for PD and DPD, and $\lambda_{EX}/\lambda_{EM}$ = 275/320 nm for IDES and DES, injection volume 20 μL.

The values of slope, calculated by linear regression (i.e. area/unit of concentration, response factor, RF), are 1315756 for PD (r=0.999) and 1179860 for DPD (r=0.999) at $297_{EX}/400_{EM}$ nm. In the case of IDES, the dependence of relative fluorescence response on λ_{EX} and λ_{EM} was measured, and the results are in Fig. 3. It can be seen on the picture that the maximum fluorescence signal for IDES is at 275 nm for excitation wavelength, and rather surprisingly at 320 nm for emission wavelength. A similar relation was found also for DES (not depicted) with a substantial difference in the maximum signal, measured also at $275_{EX}/320_{EM}$ nm, which was approximately 25 times lower in comparison to IDES. This fact limits, substantially, fluorescence detection of DES. RF values for IDES and DES at $275_{EX}/320_{EM}$ nm are 256462 for IDES (r=0.999) and 11342 for DES (r=0.998), and similarly as for PD and DPD these values are valid for 20 μL sampling loop.

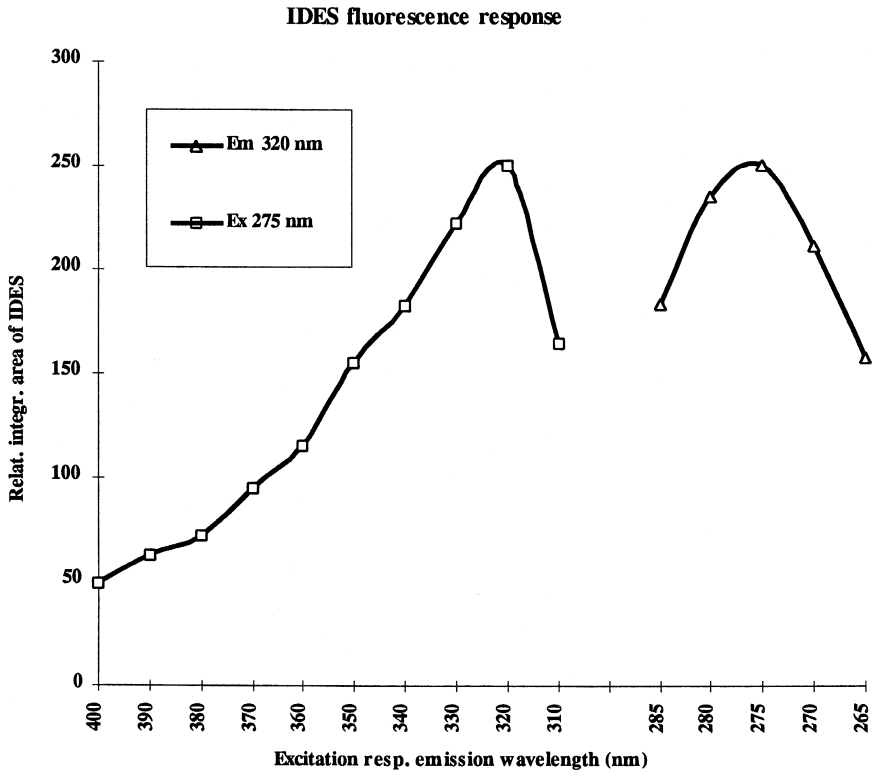

Figure 3. Relation of the sensitivity of IDES fluorescence response to the excitation or emission wavelength.

It is possible to see on Fig. 2 that the signal for IDES is ≈ 5 times lower than for PD. This fact in connection with its approximately 20 times lower concentration in urine (published results and own results for controls) means that this drawback has to be compensated by treating the samples approximately 100 times more concentrated. Due to this high concentrating of urine samples it is very important that the linear dependence of fluorescence on PD concentration is valid until 28 μg/mL (the usual concentration of pyridinoline in urine is ≈ 0.25 μg/mL). During separate HPLC runs of IDES and DES standards it appears, that they both have almost identical retention times in the separation system used. It was not possible to separate the two substances from each other, neither by any other modification of the composition of mobile phase used. This result is also demonstrated on Fig. 1, where there is only one peak corresponding to the retention time of IDES standard, although in the case of elastin hydrolysate there should be a mutually comparable quantity of both

Table 1

Decrease of Fluorescence Signal of PD and DPD Related to the Change of Emission Wavelength

Ex/Em nm	PD Res. Factor Area/Conc.	DPD Res. Factor Area/Conc.	% Relat. 297/400 PD & DPD (Mean)
297/400	1315765	1179860	100.0
297/380	877615	786967	66.7
297/370	473675	424750	36.0
297/365	317099	284346	24.1
297/360	198680	178159	15.1
297/350	65130	57813	4.9

IDES and DES. Due to the fact that DES signal is approximately 25 times lower than the signal of IDES, its contribution to an error of IDES determination in the case of possible coelution represents approximately 4%. This value is comparable to the total error of the method, and, therefore, the relevant chromatographic peak can be considered as pure IDES. Furthermore, as it was already mentioned,[15,16] the two isomers occur both in tissues and in urine practically in the same quantity, and therefore, it is sufficient to detect accurately and with enough sensitivity only one of them.

Due to a considerably lower fluorescence signal and concentration of IDES it can happen that in comparison to PD, especially in urine samples with high level of pyridinoline, its signal exceeds the measurable scope of the instrument; part of the peak area is not integrated, and the concentration would be, therefore, determined erroneously. It is therefore necessary to find such combinations of $\lambda_{EX}/\lambda_{EM}$, with which the area of PD peak is integrated intentionally with lower sensitivity, both in whole. For that, it is more suitable to change the emission wavelength. The results achieved are presented in Table 1 in the form of response factors (for 20 μL loop). It is, for instance, suitable to measure at $297_{EX}/370_{EM}$ nm, when the areas of PD and DPD peaks are decreased to approximately 1/3.

Due to the fact that the aim of this work was first of all to elaborate and to standardize the methods, the application of the work is limited only to a small number of randomly selected urinary samples. The results are summarized in Table 2, and consistently with the usually published data for UPD and UDPD they are expressed in units of nmol of crosslink per 1 mmol of urine creatinine together with conversion coefficients for customarily used units for urine IDES and DES.

Table 2

Results of HPLC Analysis of Some Randomly Selected Urine Samples*

No.	Creat. (mmol/L) Urea	PD (μg/mL) Sample	DPD (μg/mL) Sample	IDES (μg/mL) Sample	PD/Creat. (nmol/nmol) Urea	DPD/Creat. (nmol/mmol) Urea	IDES/Creat.* (nmol/mmol) Urea	Age, Yrs
TH2	6.1	12.99	2.62	0.28	39.81	8.32	0.71	57
TH1	1.8	5.18	0.74	0.11	53.66	7.96	0.95	49
TL1	4.2	6.32	1.38	0.46	28.06	6.36	1.69	43
TK1	0.8	2.95	0.42	0.11	68.76	10.17	2.13	54
TK2	7.3	13.60	1.90	0.97	34.74	5.04	2.06	51
TK3	9.6	73.41	10.10	0.57	142.60	20.38	0.92	53
RD1	10.7	22.03	6.84	0.56	38.39	12.38	0.81	70
RD2	2.8	44.40	10.00	0.34	295.70	69.18	1.88	44
Cl1	24.7	53.60	10.99	4.87	40.47	8.62	3.05	42
KM1	5.0	7.55	1.23	0.39	28.16	4.77	1.21	56
RD3	3.8	27.46	8.27	0.30	134.76	42.16	1.22	20
RD5	3.7	10.17	2.69	0.33	51.26	14.08	1.38	58
RD4	1.2	14.89	4.67	0.24	231.39	75.38	3.09	33
C12	7.8	11.22	8.52	0.96	26.82	21.16	1.90	44
C13	4.0	12.61	3.18	1.13	58.79	15.40	4.37	41
KM2	10.1	19.97	3.16	0.60	36.87	6.08	0.92	51
TK4	8.3	52.92	9.85	0.93	118.90	22.99	1.73	57
TK5	5.6	21.91	5.82	0.81	72.96	20.13	2.24	28
TH3	8.8	22.84	4.42	0.50	48.40	9.73	0.88	49
TH4	5.8	15.84	3.08	0.34	50.93	10.29	0.91	57
TL2	4.8	7.18	1.44	0.40	27.89	5.81	1.29	65
TL3	4.1	9.29	1.52	0.29	42.25	7.18	1.09	60
Cl4	6.9	19.83	3.60	1.62	53.59	10.11	3.63	49
Ol4	2.6	37.08	10.56	0.24	265.95	78.67	1.43	10
RAJ1	5.9	72.23	17.39	1.02	228.30	57.09	2.68	7
Sl1	4.9	12.29	1.96	0.67	46.77	7.75	2.12	64
MM1	1.8	3.40	1.19	0.16	35.22	12.81	1.38	44
MM2	3.3	11.21	2.85	0.21	65.35	16.73	0.98	75
RA1	3.4	24.68	4.14	0.62	135.40	23.62	2.82	48
RA2	14.2	16.19	4.08	0.27	21.36	5.63	0.29	36
RA3	10.0	11.98	3.39	1.45	22.32	6.61	2.24	44
RA4	6.5	5.92	1.11	0.46	17.08	3.34	1.10	46

* IDES/Creat.: 1 nmol/mmol = 4.57 mg/g = 8.5 pmol/mg.

It is possible to say that the values found are in conformity with published data,[3,28] but their comparison with the clinical state of the patients is not within the scope of this work. The abbreviations TH, TK, and TL correspond to the patients with transplantation of heart, transplantation of kidneys, and transplantation of liver. RD means renal dystrophy, CI cirrhosis, KM cardiomyopathy, OI osteogenesis imperfecta, MM multiple myeloma, SI skin infection, RA or RAJ rheumatoid arthritis or its juvenile form. It should be noted, that the average IDES values in nmol/mmol creatinine are: CI 3.24, KM 1.06, RD 1.68, TL 1.36, TH 0.86, TK 1.82, and RA 1.61.

Table 3

Determination of PD, DPD and IDES in Samples from Aorta and Lung of Rats and in Elastin from Bovine Ligament

		μg/mL	mg/g Dry Weight
E Elastin 5 mg/mL	IDES	24.000	4.800
A Aorta 17.5 mg/mL	IDES	7.250	0.410
	PD	1.340	0.085
	DPD	0.056	0.003
L Lung 100 mg/mL	IDES	5.410	0.054
	PD	3.900	0.040
	DPD	0.107	0.001

In the case of transplantations apparently enough time elapsed from the surgery, and, therefore, a possible traumatic intervention into the organism did not cause a more substantial increase of elastin tissue metabolism. In the case of severe affections of these organs it is possible to observe a more significant increase of elastin metabolites in urine only in cirrhosis. However, the number of urine samples is so small that these values cannot be considered representative for certain group of patients.

Results of HPLC analysis of tissues and elastin are presented in Table 3. In the case of elastin, only isodesmosine was determined. In the case of aorta and lung, besides isodesmosine also pyridinoline and deoxypyridinoline were measured. A demonstration of relevant chromatogram is in Fig. 4. Since DPD is considered to be a specific marker of bone resorption, its occurrence in tissue samples is rather surprising. Owing to the considerable quantity of processed sample it is necessary to mention that DPD peak on Fig. 4 is very small, so the quantity found is almost trace, nevertheless reliably detectable (cf. Tab. 3).

In the case of determination of both pyridinolines, only this type of detection is utilized due to the high sensitivity of measurement of their natural fluorescence.[29] On the contrary, in the case of determination of both desmosines usually UV-absorption is measured, although they have natural fluorescence also.[26] In this work fluorescence detection was used for the determination of all four crosslink elements, because it is usually far more sensitive than UV-absorption. However, it appeared that DES is practically not detectable by fluorescence detector, and therefore a mutual comparison of both detection principles was assayed.

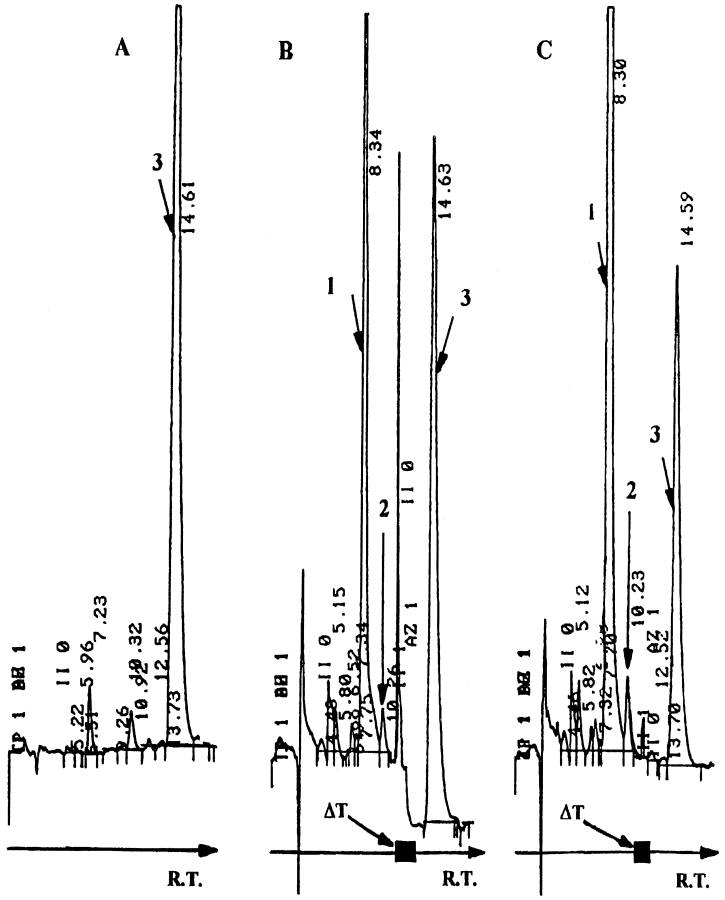

Figure 4. Record of HPLC analysis of tissue samples: A - elastin from bovine ligament (Sigma-Aldrich), 5 mg/mL, B - rat aorta, 17.5 mg of dry weight / mL, C - rat lungs, 100 mg of dry weight / mL, 1 - pyridinoline (PD), 2 - deoxypyridinoline (DPD), 3 - isodesmosine (IDES). ΔT - time interval for the change of parameters of integrator and fluorescence detector.

First of all the absorption spectrum of both IDES and DES standards was measured and flat maximum values for DES at 270 nm and for IDES at 280 nm were found (not shown). However, the differences in absorbency in the interval of 270 - 275 nm for DES and 275 - 280 nm for IDES are very small (approximately 5 % for DES and 10 % for IDES), and therefore the value of 275 nm was used for both substances, which is in conformity with already previously found optimum value for their excitation wavelength.

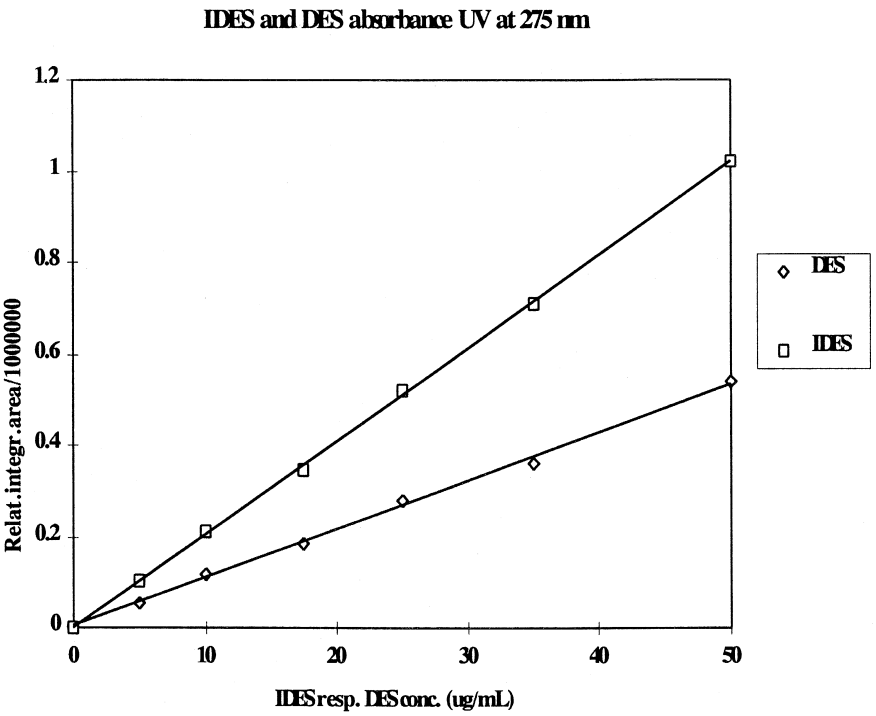

Figure 5. Calibration curves for UV-absorption of IDES and DES, expressed as the value of integrated peak area related to the concentrations. Measured at 275 nm, injection volume 20 μL.

It appeared that the sensitivity of the measurement of UV-absorption for IDES is approximately 2 times higher than for DES, as it can be seen from the measured calibration dependence on Fig. 5. The RF values (see above), calculated by linear regression, were 10692 for DES (r=0.998) and 20398 for IDES (r=0.999).

The values of relative sensitivity of both detectors used are summarized in Table 4, and they are expressed both in RF values and in relative percentages of highest sensitivity (i.e. for the fluorescence detection of pyridinoline at $297_{EX}/400_{EM}$ nm). The values for DES and IDES are set for fluorescence at $275_{EX}/320_{EM}$, for UV-absorption at 275 nm. Due to somewhat better signal to noise ratio in the case of IDES and DES, the measurement of UV-absorption is more advantageous than it appears in the last column of Table 4. Therefore, IDES is in fact only approximately 5 times less sensitive (in comparison to fluorescence), while the table indicates more than 10 times worse sensitivity of

Table 4

Relative Sensitivity of Fluorescence Detection of PD and DPD ($297_{EX}/400_{EM}$ nm) and IDES and DES ($275_{EX}/320_{EM}$ nm) vs. UV-Absorption (275 nm) During the Determination of IDES and DES

	Fluorescence		UV-Abosorbance	
	Area/Conc. RF	**Relat. % of Max* (PD. Fluor.)**	**Area/Conc. RF**	**Relat. % of Max.* (PD Fluor.)**
PD	1315765*	100*		
DPD	1179860	89.7		
IDES	256462	19.5	20398	1.6
DES	11342	0.9	10692	0.8

* The results are expressed as a relative integrated area, related to the unit of concentration, and also in percentages of this value, related to its maximum, corresponding to the fluorescence detection of PD $297_{EX}/400_{EM}$ nm.

this determination. It is possible to say that the fluorescence measurement is considerably more sensitive when the detectors selected by us, are used for the determination of PD, DPD, and IDES. If it is also necessary to quantify DES, then it is advisable to measure UV-absorption and of course to concentrate the urine at least 5 times.

CONCLUSIONS

Isocratic reverse phase HPLC method was elaborated, which enables simultaneous determination of pyridinoline (PD), deoxypyridinoline (DPD), and isodesmosine (IDES) in urine and in tissue samples with the use of fluorescence detection. The stationary phase is nonpolar sorbent based on silica modified by aliphatic octadecyl chain. Mobile phase is 0.02 M heptafluorobyturic acid (HFBA) as ion-pairing reagent with the addition of 0.01 M ammonium sulphate and 8 - 12 % acetonitrile (ACN) as organic modifier. The flow rate is 0.5 mL/min., temperature 40°C, retention times for PD and DPD are about 8 and 10 minutes, respectively, IDES is eluted at approximately 14 minutes, and the time of one determination does not exceed 16 min. Both desmosine isomers have practically the same retention times. However, due to little signal of desmosine, the IDES determination is not disturbed. The required volume of urine is 25 mL.

For PD and DPD fluorescence detection the usual values of excitation (λ_{EX}) and emission (λ_{EM}) wavelengths of 297 and 400 nm, respectively, were used. For IDES and DES the values of λ_{EX} and λ_{EM} of 275 nm and 320 nm, respectively, were used. IDES fluorescence detection is approximately 5 times less sensitive than in the case of PD and DPD, and the fluorescence signal of DES is practically negligible. Because of considerable imbalance between the concentration and sensitivity of fluorescence detection of desmosines and pyridinolines, it is necessary in some cases to decrease intentionally the sensitivity of pyridinoline detection.

By comparison of sensitivity of fluorescence and UV-absorption detector it was found that (with respect to the signal to noise ratio) the fluorescence detection of IDES is approximately 5 times more sensitive than its measurement by UV-absorption. UV-absorption is in the case of IDES approximately 2 times more sensitive than in DES. However, for this way of determination it would be necessary to concentrate urine samples at least 5 times more.

Several urine samples were analyzed for illustration. The values of PD and DPD correspond very well to the results obtained by independent determination with HPLC on cation exchanger (r=0.97, P=0.94). The concentration of IDES does not differ significantly from published control values. The elaborated method was modified and applied to elastin and tissue samples from rat aorta and lung. The chromatograms obtained are considerably more transparent than in urine samples.

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