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Note

Improved high-performance liquid chromatographic determination of bacterial collagenase activity in ointments

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Bacterial collagenase (Clostridiopeptidase A, E.C. 3.4.24.3) has been shown to improve the antibiotic action when added to ointments used for topical treatment of ulcers. In order to quantify collagenase activity, labelled peptides have been introduced as substrates to bypass the time-consuming assays which use collagen as a substrate[1–3]. The rapid and simple spectrophotometric method of Wunsch and Heidrich[4] uses the N-protected pentapeptide 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (PZ-peptide), which is hydrolysed by collagenase to PZ-Pro-Leu and then quantified at 320 nm. However, the presence in the tested ointments of compounds with chromophoric groups with a maximum absorption wavelength near 320 nm makes the spectrophotometric procedure unsuitable for collagenase assay. Recently, we devised a procedure to quantify accurately PZ-Pro-Leu, produced from PZ-peptide, by high-performance liquid chromatography (HPLC)[5]. In this study the method previously described was slightly changed and its suitability for collagenase analysis in ointments, where interfering substances are present, was tested.

EXPERIMENTAL

Materials

PZ-peptide, PZ-Pro-Leu and PZ-Pro-Phe were supplied by Serva (Heidelberg, F.R.G.). Ointments, containing collagenase and different antibiotics, were used as model formulations. Iruxol, containing collagenase and chloramphenicol, was supplied by Knoll (Liestal, Switzerland); ointments containing variable amounts of collagenase and rifamicin SV were a gift from Lepetit (Anagni, Italy).

A 2.5-m*M* solution of PZ-peptide was prepared by dissolving it in 2% final volume of methanol and diluting with 50 m*M* Tris–HCl (pH 7.5)–0.1 *M* sodium chloride–10 m*M* calcium acetate. This solution was prepared weekly and kept at −20°C when not used. The purity of PZ-peptide was checked every day by HPLC under the conditions described below. No PZ-Pro-Leu or any other spurious peak due to non-specific hydrolysis was detected even after repeated freezing and thawing cycles. A 0.1 m*M* solution of internal standard (PZ-Pro-Phe) was prepared in the HPLC eluent and maintained at 4°C.

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Chromatographic conditions

A Jasco (Tokyo, Japan) HPLC system, equipped with a Twincle pump and an Uvidec 100-III detector, set at 320 nm, was used. The a.u.f.s. setting of the detector varied between 0.01 and 0.04 depending on collagenase activity tested. Analyses were carried out on a cartridge (25 cm × 4 mm I.D.) packed with LiChrospher 100 RP-18 (5 μm) (Merck, Darmstadt, F.R.G.). A mixture of 0.1 *M* phosphoric acid (adjusted to pH 3 with triethylamine)–acetonitrile (40:60) was used as the eluent at a flow-rate of 0.8 ml/min.

Procedure

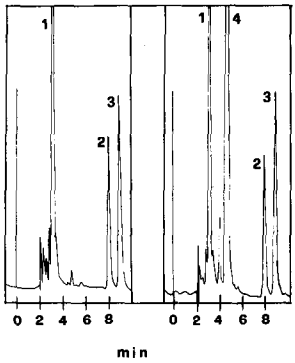
About 0.5 g of ointment in a screw-capped vial was extracted by end-over-end mixing for 30 min at 4°C with a mixture of 50 m*M* Tris–HCl (pH 7.5)–0.1 *M* sodium chloride–10 m*M* calcium acetate (2 ml) and light petroleum (b.p. 40–60°C) (3 ml). The aqueous phase was filtered using a Millipore HV 0.45-μm filter and 200 μl were kept at 25°C for 10 min. The enzymatic reaction was started with the addition of 2.5 m*M* PZ-peptide solution (50 μl). At timed intervals (5, 10 and 15 min), 50-μl aliquots were withdrawn and added to 100 μl of 0.1 m*M* PZ-Pro-Phe solution in the HPLC eluent mixture to stop the enzyme activity; 10 μl were finally analysed by HPLC.

PZ-Pro-Leu, produced by enzymatic hydrolysis, was quantitated from the peak height ratio PZ-Pro-Leu/PZ-Pro-Phe and the calibration graph obtained with the pure peptides, according to the procedure previously described[5].

RESULTS AND DISCUSSION

We introduced some slight modifications to the procedure recently described[5] in order to improve the sensitivity and the reproducibility of the method and to make it suitable for the analysis of ointments containing interfering compounds, like rifamycin SV. The HPLC eluent mixture was used to stop the enzyme reaction instead of aqueous citric acid. When the ointment containing rifamycin SV was analysed according to the method previously described, the final pH decrease brought about the precipitation of the antibiotic; consequently, the PZ-peptide peaks drastically decreased in the chromatographic profiles, due to their coprecipitation with rifamycin SV. In contrast, the presence of acetonitrile in the final mixture prevents any precipitation of PZ-peptide and assures total inactivation of the enzyme. The pH was increased from 7.0 to 7.5 following the study of the dependence of collagenase activity on pH, which showed that the maximum activity occurs at pH 7.5.

Another difference between the two procedures is the addition of the internal standard PZ-Pro-Phe at the end of enzyme incubation instead of at the beginning. The methanol used to make up standard stock solutions can cause a partial inactivation of the enzyme when added to the incubation mixture. Addition of the internal standard solution after the incubation allows the final methanol content in the assay mixture to be lowered to 0.4%, eliminating any possible interference with the enzyme action. The PZ-peptide content in the final reaction mixture was lowered from 1 to 0.5 m*M* to prevent partial precipitation when an extract of ointment containing rifamycin SV was analysed. It appears that PZ-peptide and rifamycin SV can interact also at neutral pH in highly concentrated aqueous solutions to give a precipitate which does not allow the enzyme incubation to be homogeneous. Fig. 1 shows two typical chro-



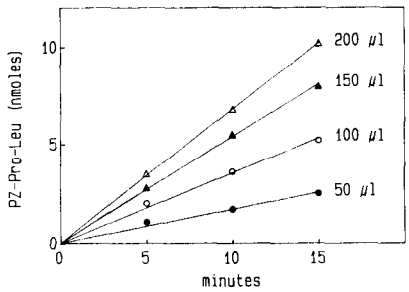


Fig. 1. Typical HPLC profiles, obtained from ointments containing chloramphenicol (left) or rifamycin SV (right), treated according the procedure described. Peaks: 1 = PZ-peptide; 2 = PZ-Pro-Leu; 3 = PZ-Pro-Phe; 4 = rifamycin SV.

Fig. 2. Time dependence of the production of PZ-Pro-Leu with different volumes of ointment extract.

matographic profiles obtained after collagenase extraction from ointments containing chloramphenicol or rifamycin SV. The latter antibiotic appears in the HPLC profile between PZ-peptide and PZ-Pro-Leu without interfering in the quantitative analysis of enzymatic activity.

It is noteworthy that PZ-Pro-Leu can be quantified by spectrophotometric analysis alone in the presence of chloramphenicol which does not significantly absorb at 320 nm but not in the presence of rifamycin SV, which has a maximum absorption wavelength at 314 nm.

In order to verify the possible interference of ointment extract with collagenase activity, the kinetics of the enzyme incubation was controlled. In Fig. 2 the time dependence of the appearance of PZ-Pro-Leu with different volumes of ointment extract is shown to be linear. No PZ-Pro-Leu peak was detected when eluent mixture and substrate solution were added simultaneously to ointment extracts, showing that no activity was detected at time zero. From these data, a linear relationship has been demonstrated between the measured activity and the extract volume for a four-fold range of extract volume (50–200 μl). The equation of the resulting regression line was $y = 0.0034 \cdot$ extract volume (μl) $+ 0.0075$ ($r = 0.998$). The reproducibility of the method was studied by repeated analyses of samples of ointments containing chloramphenicol and rifamicyn SV. The collagenase activity contents were 8.1 ± 0.59 and 3.9 ± 0.26 mU/g (mean $\pm$ S.D., $n = 5$), respectively.

In conclusion, the superior selectivity and sensitivity of HPLC allows accurate measurements of collagenase activity even when interfering compounds are present and, therefore, other analytical procedures are inadequate. The method described appears suitable for routine determinations of the collagenase content in ointment and can be applied to stability studies and other pharmaceutical research problems.

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