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Enantioselective analysis of (*R*)- and (*S*)-atenolol in urine samples by a high-performance liquid chromatography column-switching setup

G. Lamprecht[a,*], T. Kraushofer[a], K. Stoschitzky[b], W. Lindner[a]

[a]*Institute of Analytical Chemistry, University of Vienna, Waehringerstrasse 38, A-1090 Vienna, Austria*
[b]*Karl Franzens University, Department of Medicine, Division of Cardiology, Graz, Austria*

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Abstract

An HPLC column-switching method for the enantioselective determination of (*R*,*S*)-atenolol in human urine was developed and validated. Diluted urine samples were injected onto a LiChrospher ADS restricted access column and atenolol was separated from most of the matrix components using 0.01 *M* Tris buffer. The atenolol peak was sharpened by a step gradient of 30% acetonitrile and the atenolol-containing fraction was switched onto an enantioselective column. Separation of the atenolol enantiomers was carried out on a Chirobiotic T (Teicoplanin) column using acetonitrile–methanol–acetic acid–triethylamine (55:45:0.3:0.2, v/v/v/v) as eluent. Detection of the effluent was performed by fluorescence measurement. Several experiments were carried out to suppress the high blank reading, which was efficiently achieved using Tris buffer in the first dimension. For the enantioselective analysis of (*R*)- and (*S*)-atenolol in plasma under the same conditions the sample capacity of the ADS column is considerably lower.

Keywords: Atenolol; Restricted access; Column switching; Enantiomer separation

1. Introduction

(*R*,*S*)-Atenolol (4-(2-hydroxy-3-isopropylamino-propoxy)phenylacetamide) is a selective β1-adrenoceptor antagonist [1] applied in the treatment of numerous cardiovascular disorders such as hypertension, angina pectoris, etc. As known from previous studies, the main pharmacological activities reside in the (*S*)-enantiomer [2,3] and therefore it is mandatory to analyse the individual enantiomers in body fluids to arrive at convincing pharmacokinetic and pharmacodynamic results.

Several methods have been reported in the literature for the enantioselective assay of (*R*)- and (*S*)-atenolol in body fluids [4–7]. A comprehensive overview of this subject was given by Egginger et al. [8]. The direct injection of biological samples, in particular of serum or plasma samples, onto the chromatographic column, often a reversed-phase (RP) column, without any sample preparation, is in most cases problematic and may lead to irreversible contamination of the separation column, mainly by proteins. This effects not only the selectivity but also the column performance and efficiency, leading to unacceptable conditions. As a consequence, most of the bioanalytical methods, including the enan-

*Corresponding author.

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tioselective method, need adequate sample pre-treatment steps, which are: an extraction and analytical enrichment step — usually performed by liquid–liquid- or liquid–solid-phase extraction (SPE) — followed by the direct enantioseparation of the pretreated sample on a chiral stationary phase (CSP) or by an indirect approach involving derivatisation with a chiral reagent and the analysis of the pair of diastereomers on, for example, a RP system.

The objective of the present work was to develop an on-line sample preparation and analysis method to be used for the analysis of (*R*)- and (*S*)-atenolol in urine and, possibly, also in plasma. A powerful tool to accomplish this relies on the implementation of restricted access material (RAM) for sample preparation [9–11]. The RAMs and particles, respectively, are characterised by a hydrophilic outer surface, a small pore size and a lipophilic inner surface. Proteins of high molecular weight cannot penetrate into the pores, thus being excluded on the hydrophilic outer surface. Hence they can easily be separated from sample matrices consisting of small molecules, including drug compounds and metabolites [12].

The present paper describes the elaboration of an enantioselective HPLC column-switching method for (*R*)- and (*S*)-atenolol RAMs for on-line sample preparation and a Teicoplanin-derived chiral column to be applied for final analysis.

2. Experimental

2.1. Chemicals

For chromatography, Lichrosolv acetonitrile and methanol were purchased from Merck (Darmstadt, Germany) and racemic (*R*,*S*)-atenolol from Schweizerhall (Basel, Switzerland). All other chemicals were of analytical grade. Enantiomerically pure (*S*)-atenolol (ee >98%) and (*R*)-atenolol (ee >99%) were prepared according to a previously published method [13].

2.2. Biological samples

The urine samples represent aliquots of morning urine collected the following morning after the intake of two times 25 mg, or two times 50 mg (morning and evening) and one time 100 mg (noon) of (*R*,*S*)-atenolol (pharmaceutically formulated). The total amount of morning urine was not measured, and the volunteers did not follow any specific diet after drug intake. The dosing and sampling protocol was considered preliminary with the main focus to receive authentic human urine samples to check the practical validity of the analysis method.

2.3. Instrumentation

The total analysis concept is based on a two-dimensional chromatography system pre-separating the majority of the matrix components from the analytes which are retained on a RAM column followed by a solvent switch and fraction transfer onto the enantioselective column by means of column switching (Fig. 1). In the first dimension a high-pressure gradient pump (Model L-6200A, Merck) was used.

Samples were introduced into the chromatographic system by an injection valve (Model 7125, Rheodyne, Berkeley, CA, USA) equipped with a 20 μL injection loop. An ADS-LiChrospher RP-18 (Merck) restricted access column of dimensions 25×4 mm I.D. and 25 μm particle size was used as the separation column. Column switching was performed by means of an electrically driven switching valve (Model ELV 7000, Merck, Vienna, Austria). Detection of the column effluent was performed by a fluorescence detector (Model F1080, Merck) at an excitation wavelength of 230 nm and measuring the emission at 310 nm.

Eluent for the second dimension was delivered by a high-pressure pump (Model L6000, Merck). Separation of the transferred fraction was achieved on a Teicoplanin enantioselective column (Chirobiotic T, Astec, Whippany, NJ, USA) of dimensions 250×4.6 mm I.D. The column was thermostatted at 25°C by a column thermostat (Thermotechnik Products, Langenzersdorf, Austria). Detection of the eluate was performed by a Jasco fluorescence detector (Model FP-920, Jasco, Tokyo, Japan) at the wavelength settings mentioned above. The detector signal of both detectors was digitised by a Merck Interface (Model D-6000A). Data acquisition and processing

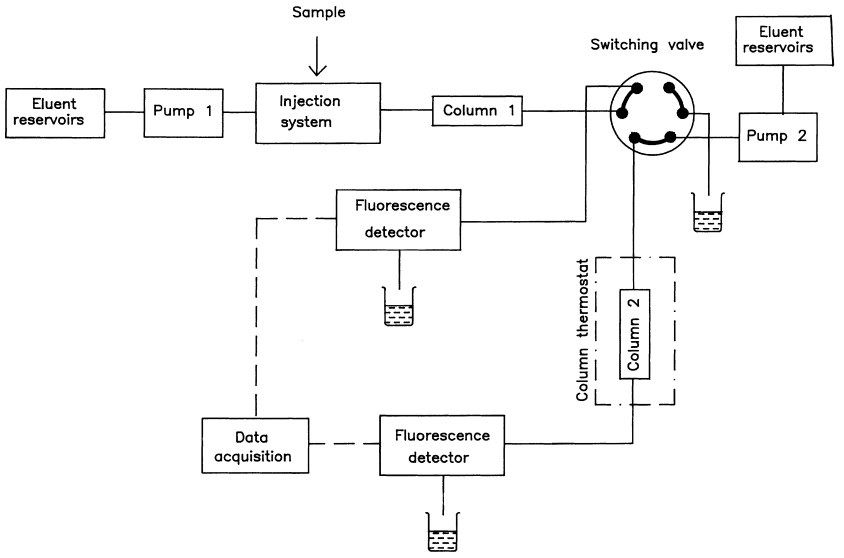

Fig. 1. Schematic of the two-dimensional chromatography system. Column 1: ADS; Column 2: Teicoplanin. For mobile phases and time events, see Table 1.

was performed by use of chromatographic software (Merck).

2.4. Chromatographic conditions

For separation in the first dimension, aqueous 0.01 *M* Tris buffer, adjusted to pH 5.0 with acetic acid, was used. After elution of the polar and unretained compounds, a step gradient of 30% acetonitrile was applied for compression of the atenolol peak. The eluent of the enantioselective column consisted of acetonitrile–methanol–acetic acid–triethylamine (55:45:0.3:0.2, v/v/v/v). For both dimensions the timed events of the chromatographic programme are summarised in Table 1.

2.5. Sample preparation and operation

Urine samples were stored at −20°C. Before analysis they were centrifuged at 15 000 *g* for 2 min and the clear supernatant was used. Samples containing large amounts of atenolol were diluted with Tris buffer (1:1, v/v) after the first analysis.

Table 1
Column-switching time table and events[a]

Time (min)	Event
0.0	Injection of 20 μL of sample; start of programme; pump 1, column 1: eluent 100% A, flow-rate 1.0 mL/min
2.3	Step gradient for peak compression on column 1: eluent 70% A+30% B
3.0	Column 1 connected to column 2, eluent 70% A+30% B
6.2	Column 2 disconnected, column 1 continued flushed
6.3	Start of pump 2; column 2: eluent 100% C; flow-rate 1.5 mL/min
8.0	Reequilibration of column 1: eluent 100% A
25.0	Pump 2 stopped; end of cycle

[a] Eluents: (A) 0.01 *M* Tris buffer pH 5.0, (B) acetonitrile, (C) acetonitrile–methanol–acetic acid–triethylamine (55:45:0.3:0.2, v/v/v/v).

3. Results and discussion

3.1. Chromatography in the first dimension (ADS column)

(*R*,*S*)-Atenolol is a highly hydrophilic drug and elutes on an ADS column under isocratic conditions as a broad peak. On a new ADS column the peak volume of atenolol with an aqueous 0.01 *M* Tris buffer is 10 mL (peak maximum at 15 mL elution volume), which is too high for an effective on-line column-switching protocol. Therefore, peak compression is necessary in order to facilitate the quantitative transfer of the compound in a small effluent volume. A sufficient peak sharpening effect can be achieved by a step gradient of 30% acetonitrile or 50% methanol.

At the start of elution of atenolol the compound is adsorbed in a broad zone on the ADS column covering nearly the whole column bed. The application of a backflush technique offers no real advantage in this case, but only increases system complexity and the possibility of the transfer of more retained compounds, adsorbed on top of the column, onto the enantioselective column.

Gradient formation was initiated about 1 min before atenolol begins to elute from the ADS column and was checked at regular intervals. Generally, we found that the start point and composition of the step gradient are not critical for a new column, whereas on an aged column the shape of the compressed peak depends on both parameters. The reason for this may be found in the fact that the surface of the separation material is altered by the urine matrix and the influence of additional interactions increases.

Under the above conditions a transfer efficiency of 99.8% for (*S*)-atenolol and 96.3% for (*R*)-atenolol ($n = 3$) was obtained.

3.2. Chromatography in the second dimension (Teicoplanin column)

3.2.1. Enantioseparation

The chiral separation of the transferred and unresolved (*R*)- and (*S*)-atenolol fraction was carried out on an enantioselective chirobiotic T column in polar organic mode applying acetonitrile–methanol–acetic acid–triethylamine (55:45:0.3:0.2, v/v/v/v) as eluent. Representative chromatograms of the standard and urine samples are shown in Fig. 2. Resolution and retention of the chiral compounds have to be controlled by temperature as a marked dependency has been observed, which is summarised in Fig. 3. Further parameters are the amount and ratio of acid to base in the eluent. Taking the above mixture as a reference, resolution and retention increased with increasing portions of acetic acid. Increasing the amount of both acid and base leads to a decrease of resolution and retention. The best resolution was obtained at an acid-to-base ratio of 4:1. The retention of both enantiomeric forms decreased, as expected, with increasing temperature (Fig. 3), but showed no significant influence on the resolution, although α decreased. Resolution R_s increased with decreasing flow-rate, resulting in values of 2.1 for 0.8 mL/min and 1.3 for 2.0 mL/min.

3.2.2. Blank reading

The individual analysis of (*R*)- and (*S*)-atenolol with the described chromatographic system was complicated during the development process due to the fact that a considerable blank reading was observed initially. Applying sodium acetate buffer as eluent in the first dimension, a reproducible blank reading of the order of 1.7 μg/mL was obtained for each enantiomer of atenolol in the second dimension. The ADS column was identified as the source of this problem and the blank reading was constant and independent of the applied matrix (e.g., plasma, urine). Even excessive cleaning of the column with organic eluents such as acetonitrile or methanol resulted in no blank reduction. It was concluded that strong adsorption of the basic analytes ($pK_a > 9.5$) was responsible, which may be related to acidic silanol functions quasi acting as a non-selective ion-exchange system. A pronounced reduction of silanol activity was achieved by lowering the pH of the eluent from 5 to 4, resulting in an 80% reduction of the blank reading. The addition of small amounts of competitive inorganic cations to the eluent, such as potassium or calcium salts, nearly completely reduced the blank reading. However, these salts also suppressed the enantiomeric separation of (*R*)- and (*S*)-atenolol on the Teicoplanin column, thus forcing us to search for another compromise.

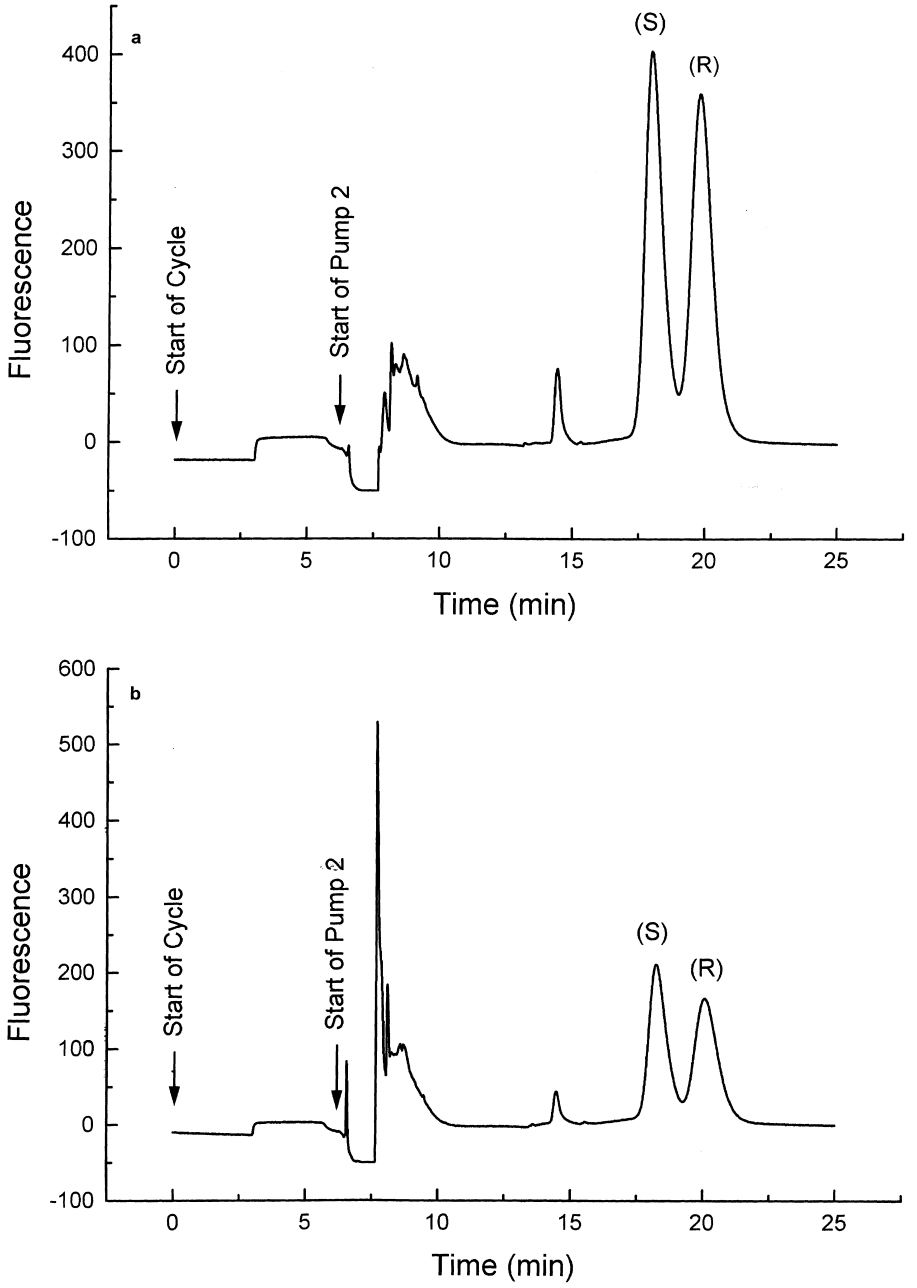

Fig. 2. Separation of switched fraction of (a) racemic atenolol standard and (b) urine sample on Chirobiotic T. Conditions as described in Section 2.

The addition of triethylamine, which is commonly used in chromatography for suppression of silanol activity, caused a further 10% reduction of the blank reading at pH 5 and about 20% at pH 4. Finally, tris(hydroxymethyl)aminomethane proved the most effective additive and removed the blank reading completely and showed no negative influence on enantiomer separation on the Teicoplanin column.

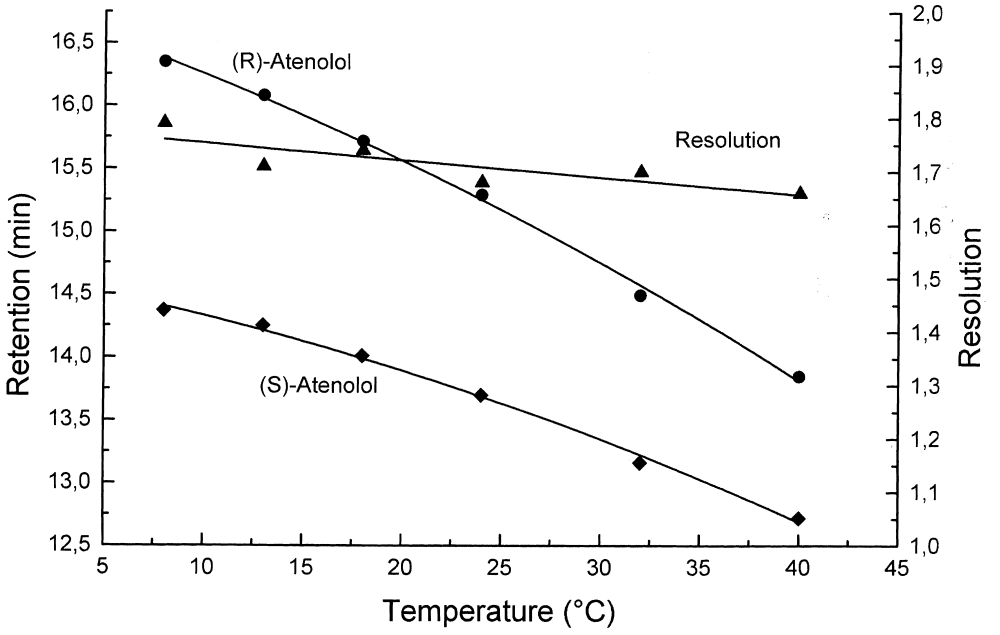

Fig. 3. Retention and resolution of (*R*)- and (*S*)-atenolol on Chirobiotic T as a function of temperature. Conditions: eluent, acetonitrile–methanol–acetic acid–triethylamine (55:45:0.3:0.2, v/v/v/v); flow-rate, 1.5 mL/min.

3.3. Application of the method to urine samples

More than 500 urine samples were analysed on the described column-switching system with only slight deterioration of column performance. However, the overall sample capacity seems higher.

The levels of (*R*)- and (*S*)-atenolol in urine of volunteers after oral administration of different amounts of racemic atenolol are shown in Table 2. From a pharmacokinetic point of view there is no significant stereoselectivity in excretion to note, which is not surprising as atenolol is only metabolised very slightly and thus excreted as the parent drug. However, the atenolol levels in serum were significantly different and higher values for (*R*)- over (*S*)-atenolol have been measured after p.o. intake of (*R*,*S*)-atenolol [2].

Table 2
(*S*)- and (*R*)-atenolol in urine after oral intake of 25–100 mg racemic atenolol

Dose	Subject	(*S*)-Atenolol (μg/mL)	(*R*)-Atenolol (μg/mL)	Ratio (*S*)/(*R*)
0 mg	1	0.0	0.0	–
	2	0.0	0.0	–
2×25 mg	3	0.82	0.84	0.972
	4	6.25	5.15	1.062
2×50 mg	5	2.35	2.24	1.049
	6	4.23	4.32	0.980
100 mg	7	4.86	4.83	1.012
	8	2.35	2.24	1.049

3.4. Application of the method to plasma samples

The same method was also applied to plasma samples. The excluded proteins of high molecular weight elute within 5 mL of 0.01 *M* Tris buffer and after 6 mL a step gradient was applied.

In contrast to the injection of urine samples we found that the ADS column performance was drastically reduced after several injections of 50 μL plasma. The reason for this may be that components of the plasma matrix below the 15 kDA exclusion limit can still penetrate into the pores and undergo adsorption, thus changing the property of the ADS material substantially and make it more polar. This slight alteration of the column surface has a strong effect on the weak interactions of atenolol inasmuch

as they are further reduced resulting in very low retention. Cleaning of the column with an eluent of low organic modifier concentration for a longer period of time prior to gradient elution — as described in the literature [9] — is not practicable because of the hydrophilic nature of the analyte. As a consequence, the described separation system could only be applied for the quantitative determination of atenolol in plasma in a very limited number of samples. No routine method could be installed with this setup.

3.5. Validation

3.5.1. Calibration

Calibration of the chromatographic system was performed with enantiomerically pure (*R*)- and (*S*)-atenolol standards in water and urine in the concentration range 0.15–15 mg/L ($n = 6$) equally distributed. In addition, urine samples were spiked with racemic atenolol. In all cases, linear calibration functions were estimated with correlation coefficients >0.9996. The corresponding regression functions were as follows. Water: (*S*)-atenolol, $y = 5.37 \times 10^{-7}x + 0.11$ ($r = 0.9999$); (*R*)-atenolol, $4.88 \times 10^{-7}x + 0.011$ ($r = 0.9999$). Urine: (*S*)-atenolol, $y = 4.95 \times 10^{-7}x + 0.095$ ($r = 0.9999$); (*R*)-atenolol, $y = 4.97 \times 10^{-7}x - 0.012$ ($r = 0.9998$). Racemic spiked urine: (*S*)-atenolol, $y = 5.20 \times 10^{-7}x - 0.045$ ($r = 0.9998$); (*R*)-atenolol, $y = 5.00 \times 10^{-7}x - 6.8 \times 10^{-3}$ ($r = 0.9997$).

Operating the detector in the most sensitive mode, a detection limit of 15 μg/L was found for each enantiomer.

3.5.2. Reproducibility

The reproducibility of the method varied between 1.9 and 4.2% for (*R*)-atenolol and between 0.8 and 3.0% for (*S*)-atenolol for 15 and 1 mg/L. For the day-to-day reproducibility, a relative standard deviation of 7.5% for (*R*)-atenolol and 3.5% for (*S*)-atenolol was calculated ($n = 5$).

3.5.3. Sample degradation

Stability of standard and urine samples was investigated at 6°C and at ambient temperature (25°C). No significant degradation was observed at 6°C within 14 days. At ambient temperature and daylight, recovery of (*R,S*)-atenolol dropped to 85% within 5

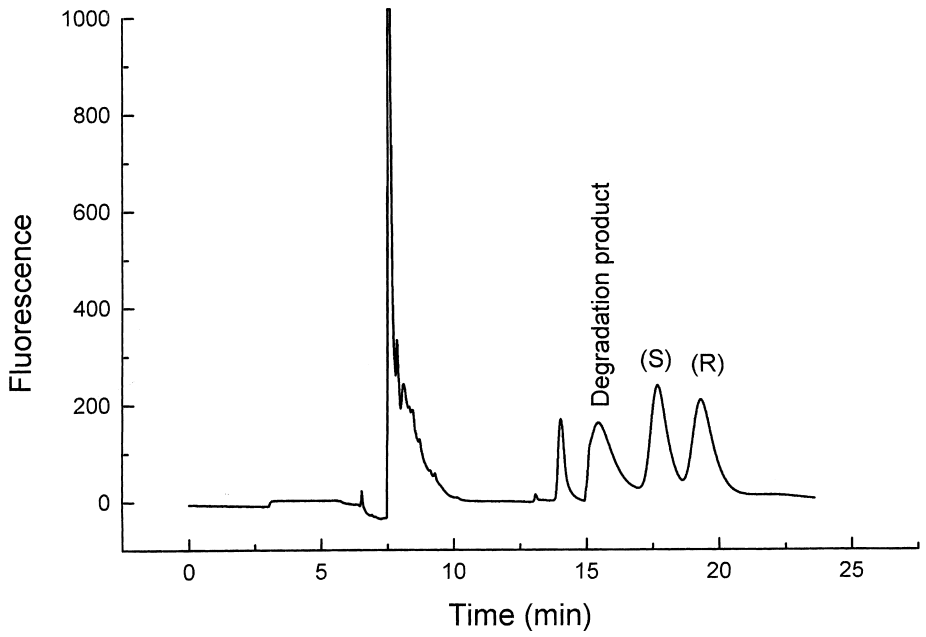

Fig. 4. Chromatogram of a degraded urine sample on Chirobiotic T. Conditions as described in Section 2.

days. Sample degradation is manifested as an additional peak in the chromatogram (Fig. 4). The peak was not identified. This observation leads to the protocol to work up and measure the urine samples within 1 day to avoid degradation.

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