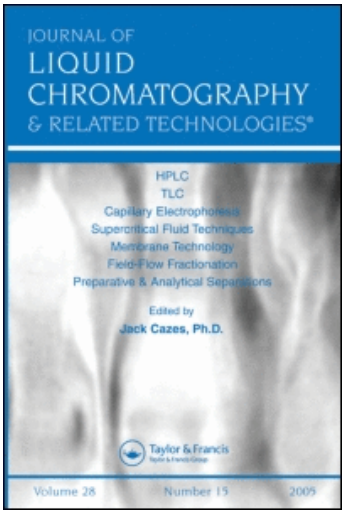

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ACCURACY IN THE DETERMINATION OF RECOVERY IN HPLC

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ABSTRACT

A method for the determination of the recovery in RP-HPLC using fluorescence and UV-detection is presented and compared with radioisotope experiments. A sensitive and specific detection is necessary in order to obtain accurate results.

INTRODUCTION

Several procedures for the determination of the recovery in reversed phase HPLC using UV-detektion have been published. Remarkably, Rossi et al.[1] found consistently that recovery values were below unity, this was discussed as being caused by the method. Welinder et al.[2] obtained variable results depending on the used procedure. Obviously the accuracy of

the recovery determined by UV-detection is a serious problem. This paper presents a procedure for the determination of the recovery using fluorescence and UV-detection and reports values of the accuracy of recovery measurements. Anisole, triiodothyronine and 2-iodoestradiol were used as model compounds. The data is compared with recovery data estimated by counting ^{125}I-activity of chemically identical species.

EXPERIMENTAL

Materials

Anisole was obtained from Berlin-Chemie AG, FRG, L-3.5.3'-triiodothyronine (T_3), reagent grade, was purchased from Hennig Berlin GmbH, FRG. 2-iodoestradiol (E_2) was obtained from the Central Institute for Isotope and Radiation Research, and was recrystallized from benzene/hexane. The chemical purity of these compounds as examined by HPLC was better than 99% . ^{125}I-labelled compounds were from the same institute and were chromatographically purified with the HPLC-system described below. The radiochemical purity was better than 99% for E_2 and about 97% for T_3. Methanol was reagent grade and additionally purified by destillation.

HPLC-System

The HPLC-system (Shimadzu Corp.,Japan) consisted of a LC-6A pump with a high sensitivity filter for pulse dampening, a sample injector Rheodyne 7125 (Rheodyne Inc.,U.S.A.), a six-port valve Rheodyne 7000 or Valco C6W for selection of flow through the column or the bypassing capillary (Fig.1) and a fluorescence detector RF-535 or a photometric detector SPD-6AV connected with an integrator C-R5A . The fluorescence was detected at an excitation wavelength of 272.5 nm

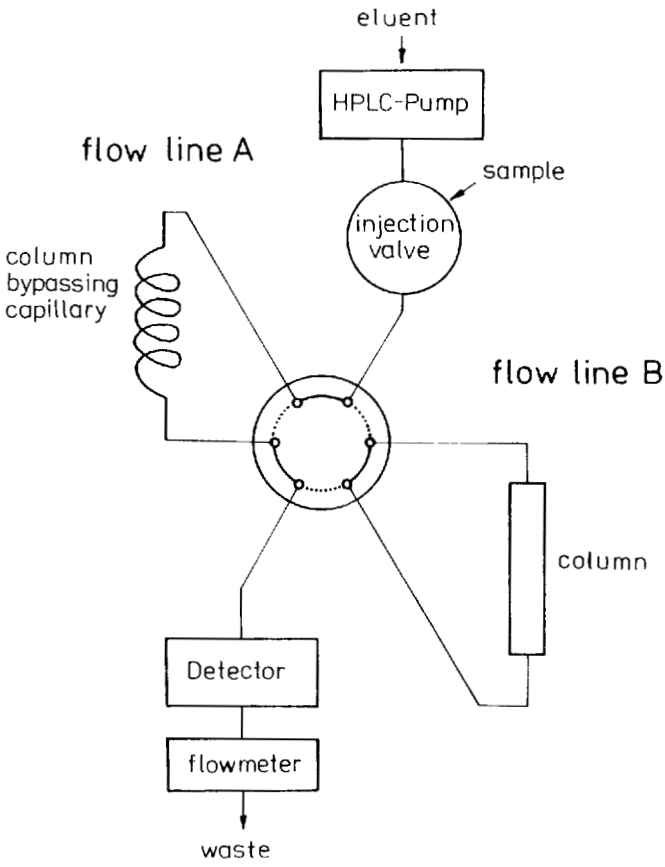

Fig. 1 Block diagram of the HPLC-apparatus

and an emission wavelength of 290 nm. The absorbance was measured at 270 nm for anisole, at 296 nm for T_3 and at 290 nm for E_2. For the integration, a sampling interval of 0.5 sec and the lowest possible slope was used to guarantee a complete integration of the tails of the peaks.

The outlet of the detector was connected to a flowmeter. The flow rate was registered simultaneously when the chromatographic peak was detected.

The recovery was investigated using analytical columns 150 * 3.3 mm packed with Separon SGX RP18, 5 μm (Tessek, Czechoslovakia). Methanol/water mixtures were used as mobile phase. For T_3 a methanol/0.1% phosphoric acid (70:30,V/V) eluent was used [3]. The flow rate was 0.5 ml/min.

Length and inner diameter of the bypassing capillary were chosen to give a peak shape similar to that observed in the chromatogram. A capillary with i.d. of 0.3 mm and a length of 3 m was used.

Procedure

The sample was dissolved in the eluent. Solvents different from the eluent were not used in order to avoid "system peaks". The following scheme for measurements was followed.

First, the sample was injected into the chromatographic system bypassing the column (flow line A, Fig.1), and the peak area representing the total amount of injected sample was measured. This measurement was repeated 1 to 3 times.

Second, the system was switched to flow line B. After the flow rate had stabilized, the sample was injected and the sum of the peak areas of the chromatogram was determined (3 to 7 repetitions).

Third, the system was reswitched to flow line A and the same number of measurements were performed. This was done to correct for a possible drift in the detector response and in changes in the concentration of the sample solution.

Special care was taken with respect to the flow rate. Data showed the necessity to correct for changes

in the flow rate caused mainly by the different flow resistances of line A (capillary) and line B (column). These corrections were performed using the known reciprocal dependence of peak area on flow rate [4].

The recovery is determined from the quotient of peak areas using the two different flow lines A and B (Fig.1). Therefore it is concluded that it is independent of all interferences which influence both flow lines to the same extent.

The recovery of added ^{125}I-tracers was measured by collecting 1 ml fractions during the entire run of the chromatogram. The fractions were counted on an automatic γ-counter (PW 4800, Philips, Netherlands). The recovery was evaluated by comparing the -activity eluted from the column and the γ-activity observed in the outflow of the injection valve. All measurements of labelled samples were preceded by injections of unlabelled samples into the same flow line.

RESULTS AND DISCUSSION

Anisole is often used as a model compound in testing reversed phase chromatographic systems [5]. The recovery of such a standard compound is expected to approach unity. The experimental data show that for a peak with an asymmetry of about 2.5 the integration has to be continued until the signal is about 0.1 percent of the maximum of the peak. Only this procedure guarantees the difference between the determined peak area and the true peak area to be lower than 1 percent. This needs a chromatographic system with a baseline noise lower than 0.05 percent of the signal at the maximum of the peak. As shown below, in this case the reproducibility of peak areas is good. The area of

the peak of anisole eluted from the column (flow line B, Fig.1) is somewhat smaller than that detected after bypassing the column (flow line A). This difference can be explained by the flow data.

The precision of fluorimetrically detected peak areas is about 0.5-1.0 percent corresponding to an error of recovery near 1.5 percent.

The good reproducibility from day to day of the described method was demonstrated in 14 experiments. For the determination of the anisole recovery we obtained a mean recovery of 1.002 ±0.007.

For testing the ruggedness of this method the experimental conditions were changed. With a flow rate of 0.3 ml/min the same recovery was found. The dependence of recovery on the shape of the peaks obtained from line A and from the column was tested by changing the eluent from pure methanol to a methanol/water mixture (60:40,V/V). Typical chromatograms are given in figure 2. After a large number of these experiments the column performance deteriorated and the peaks showed tailing. Peak asymmetry increased to 3, but the recovery was not influenced. The prerequisite was a complete integration of the observed peaks, as mentioned above. This result was confirmed by the experiments with triiodothyronine (Fig.3, Tab.2).

The measurements of anisole were done by fluorimetric detection. In this case no, or only very small "system peaks" were found. The situation was quite different using UV-absorption at a wavelength near to or smaller than 220 nm for the detection. Clear "system peaks" were observed, the recovery data was poorly reproducible from day to day and their mean was smaller than unity. Absorption measurements at 270 nm gave better results. In four experiments we obtained a recovery of anisole of 1.00±0.015 using methanol or a

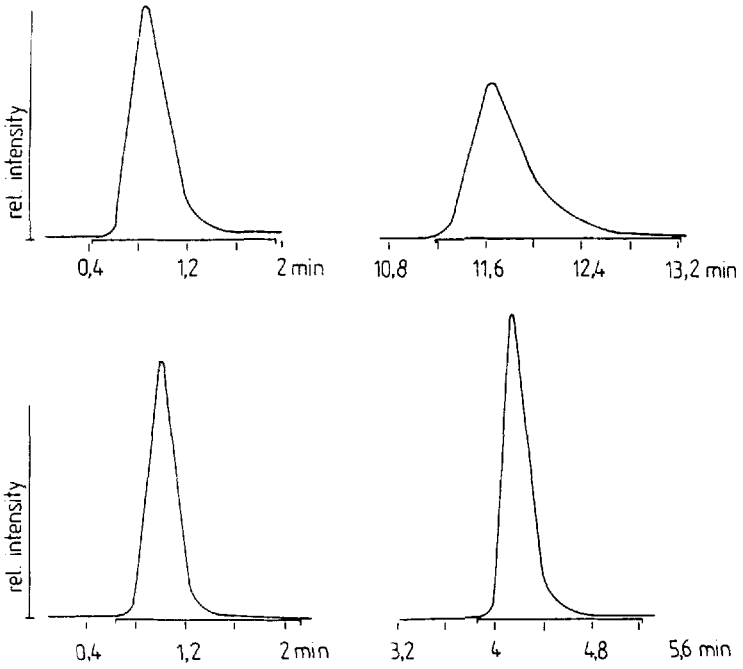

Fig. 2 Chromatogram of 4 nmol anisole detected by fluorescence,
left : the peak bypassing the column
top : eluent - methanol/water 60:40,V/V rec.= 0.996±0.005
bottom: eluent - methanol, rec.= 1.000±0.007

methanol/water mixture (60:40,V/V) as eluent. These results show that a detection method which is sensitive and specific for the considered components of the sample is more suitable for the estimation of recovery than a universal detection system such as UV-absorption at wavelengths below 230 nm.

The accuracy of the described method was estimated investigating the recovery of triiodothyronine (T_3) and 2-iodoestradiol (E_2). ^{125}I labelled tracers

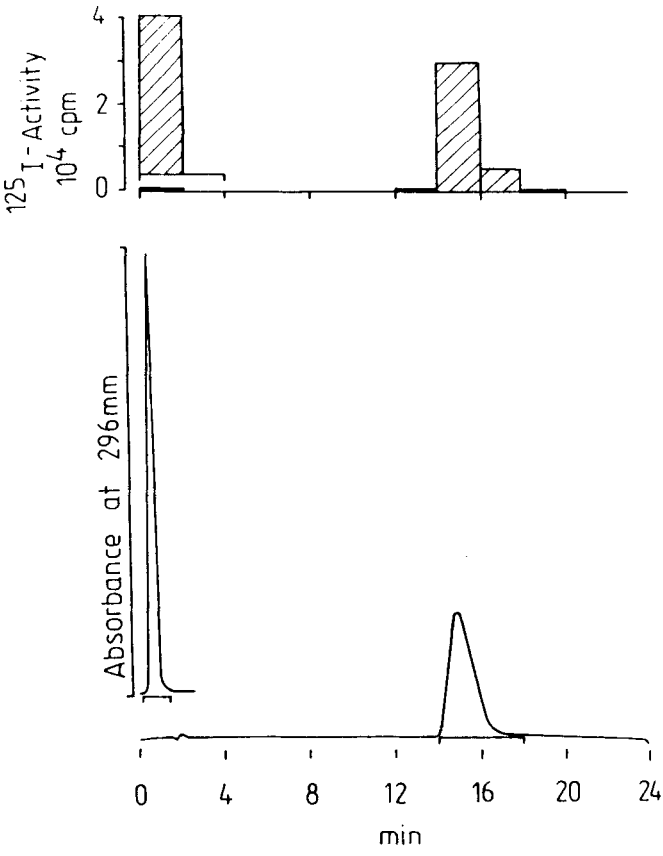

Fig. 3 Chromatograms of 8 nmol T_3 with added ^{125}I-tracer, left : the peak bypassing the column

were added to the unlabelled compounds and both, the absorbance near 290 nm and the γ-activity, were measured in one and the same experiment. The results are given in table 1, 2 and figure 3.

The fact that there was no difference between the injected γ-activity and γ-activity which had flown through the bypassing capillary is of great

TABLE 1 Recovery of Triiodothyronine with added ^{125}I-Tracer as determined by γ-counting and Absorption Measurement

Sample	γ-activity cpm	absorption at 296 nm arbitrary area units
injected	37 190 ±426	-
bypassing the column	37 448 ±326	69 816 ±213
eluted from the column	37 192 ±131	70 048 ±925
recovery	1.000 ±0.012	1.003 ±0.014

TABLE 2 Recovery of 2-iodoestradiol with added ^{125}I-Tracer as determined by γ-counting and Absorption Measurement

Sample	γ-activity cpm	absorption at 290 nm arbitrary area units
injected	146 250 ±540	-
bypassing the column	146 280 ±470	107 230 ±636
eluted from the column	146 460 ±1100	106 140 ±860
recovery	1.001 ±0.008	0.990 ±0.010

importance. This equivalence is essential for the described method of recovery determination. The recoveries determined on the basis of the injected γ-activity and evaluated using the absorption data show no difference, confirming the accuracy of the described method.

The recovery of a mixture of phenol, p-cresol, 2,5-xylenol and anisole was determined to be 1.008 ±0.011. This demonstrates that this method is applicable to complex samples.

In conclusion, the experiments described above clearly indicate that the applied procedure for the measurement of the recovery is versatile and reliable. It may be useful for the validation of chromatographic methods and for the characterization of stationary phases with respect to the recovery of special types of compounds.

REFERENCES

1 D.T.Rossi, F.Pacholec, L.M.Dawkins, Integral Method for Evaluating Component Contribution to Total Solution Absorbance from Chromatographic Data, Anal.Chem., 58 (1986) 1410-1414.

2 B.S.Welinder, H.H.Sørensen, B.Hansen, Recovery of Polypeptides after Reversed-Phase-HPLC, J.Chromatogr., 408 (1987) 191-199.

3 B.R.Hepler, S.G.Weber, W.C.Purdy, Liquid Chromatographic Separation of the Thyroid Hormones, Anal.Chim.Acta, 138 (1982) 221-230.

4 J.Asshauer,H.Ullner, Practice of HPLC, H.Engelhardt eds., Berlin 1986, 65-108.

5 P.A.Briston, J.H.Knox, Standardization of Test Conditions for HPLC Columns, Chromatographia, 10 (1977) 279-289.