Journal of Chromatography, 525 (1990) 369–377
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5061

SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF EUGENOL IN BODY FLUIDS

I.U. FISCHER and H.J. DENGLER*

Medizinische Universitatsklinik Bonn, Sigmund-Freud-Strasse 25, D-5300 Bonn 1 (F.R.G.)

(First received March 22nd, 1989; revised manuscript received September 30th, 1989)

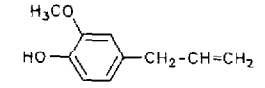
SUMMARY

The high-performance liquid chromatographic assay described permitted a simple, rapid, sensitive, selective and precise quantitative determination of eugenol in body fluids (serum, urine and bile) without derivatization. Amounts in the range 0.02–100 μg of eugenol per millilitre of body fluid were determined with intra-assay coefficients of variation below 4% (3.72–1.13%). The short analysis time for each sample and the selectivity even at low concentrations made this assay suitable for pharmacokinetic studies. Eugenol undergoes a pronounced first-pass effect; in serum, unconjugated eugenol was not detected after an oral dose of 150 mg. The kinetics of eugenol conjugates were measured. More than 80% of the dose was excreted within 6 h after oral administration.

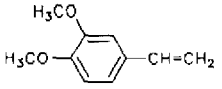
INTRODUCTION

Eugenol (4-hydroxy-3-methoxyallylbenzene, I, Fig. 1) is widely distributed in the plant kingdom, mainly as a constituent of essential oils [1,2]. It is used principally as a fragrance and flavouring agent, as an analgesic in dental materials and non-prescription drug products, as an insect repellent and as a chemical intermediate [2,3]. Daily human per-capita consumption of eugenol was estimated to have been 0.6 mg [1]. The Joint Food and Agriculture Organization/World Health Organization (WHO) Expert Committee on Food Additives established a conditional acceptable daily intake of eugenol for humans of 0–5 mg/kg body weight (WHO 1967) [1]. As for other phenols in essential oils, eugenol can be determined after saponification by extraction with aqueous sodium hydroxide solution [2]. This approach does not distinguish between different phenols, hence qualitative [4] and quantitative [5–7]

0378-4347/90/$03.50



I



II

Fig. 1. Structures of eugenol (I) and 3,4-dimethoxystyrene, the internal standard (II)

gas chromatographic (GC) assays have been developed for eugenol together with other phenols present in essential oils. GC assays have also been applied for the determination of eugenol together with other allylbenzenes [8–12]. In all these cases, reliable separation and/or quantification was required. Because of the relatively large and concentrated samples available, sensitivity was of minor concern.

Because of the lack of chromophoric groups in compounds such as the monoterpene hydrocarbons and alcohols, many essential oil constituents cannot be analysed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. For eugenol, a phenylpropane derivative, the HPLC assays described have employed reversed-phase columns and UV detection at different wavelengths [13–15]. This report describes a simple, rapid, selective and sensitive HPLC method for quantitative determination of eugenol in human serum, urine and bile.

EXPERIMENTAL

Volunteers and patients

Six young, healthy volunteers (four females, two males, 23–29 years, body weight 52–86 kg) participated in a pharmacokinetic study. The liver and kidney functions were normal according to the appropriate clinical chemistry parameters. Each volunteer took three gelatin capsules each containing 50 mg of eugenol together with a 'standard breakfast'. The 24-h urine was collected in 0–3, 3–6, 6–12 and 12–24 h intervals. Venous blood samples were obtained by an indwelling cannula at 0, 15, 20, 25, 30, 40, 50, 60, 80, 100 and 120 min. Bile samples (6 h) were obtained from patients with indwelling T-tubes (Department of Surgery, University of Bonn, Bonn, F.R.G.). These patients also volunteered to take three capsules containing 50 mg of eugenol each.

Materials

Eugenol (I, Fig. 1), the sulphuric acid ester of eugenol, and also the eugenol-filled capsules were a gift from M.C.M. Klosterfrau (Cologne, F.R.G.). 3,4-Dimethoxystyrene (II, Fig. 1), used as internal standard (I.S.), was purchased from Aldrich (Steinheim, F.R.G.). HPLC-grade acetonitrile and methanol were obtained from Baker (Deventer, The Netherlands). All other reagents were analytical grade. Nucleosil C_{18}, particle size 10 μm, for the column was purchased from Macherey-Nagel (Düren, F.R.G.) and the Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.). The enzyme preparation β-glucuronidase/arylsulphatase (from *Helix pomatia*, activity 5 and 14 U/ml) was purchased from Serva (Heidelberg, F.R.G.).

Equipment

Analyses were performed on a 30 cm $\times$ 4.0 mm I.D. stainless-steel column packed with Nucleosil C_{18}, particle size 10 μm. The detector was a Shimadzu SPD-6A UV spectrophotometer monitoring at 220 nm. The eluent flow was generated by a Shimadzu LC-6A pump (Kyoto, Japan). Injections were made using a syringe loading injector from Rheodyne (Cotati, CA, U.S.A.). Recording was done with a Servogor 210 recorder from BBC Metrawatt (Nürnberg, F.R.G.). The mass spectrometer was an LKB 2091 (Bromma, Sweden) coupled with a Carlo Erba 4180 gas chromatograph (Milan, Italy). A Carlo Erba 4180 gas chromatograph with a flame ionization detector was used for the GC measurements.

Methods

In the general extraction method for untreated or enzymically hydrolysed samples, 10 μl of the required I.S. solution (20 ng/μl or 0.5 μg/μl of methanol) were added to a 0.5-ml aliquot of serum, urine or bile. In the case of serum or urine, the aqueous phase was mixed and extracted with 3 ml of *n*-hexane by shaking for 1 min on a mixer. The samples were centrifuged at 2500 *g* for 10 min. The organic phase was transferred to a clean conical glass tube and evaporated in a nitrogen stream at room temperature (20 ± 3°C) just to dryness.

For enzymic cleavage of the conjugates, 0.5 ml of sodium acetate buffer (0.1 *M*, pH 4) and 20 μl of β-glucuronidase/arylsulphatase were added to each sample. After incubation for 5 h at 37°C, 150 mg of ammonium sulphate were added to stop the enzymic reaction. The incubates were extracted as described above.

An additional purification step was used for bile samples. After incubation the interfering compounds were separated with Sep-Pak C_{18} cartridges. Each incubated solution was applied to a cartridge, and the interfering compounds were washed off the cartridge. Eugenol and the I.S. remained on the cartridge and were eluted with 3 ml of *n*-hexane. The solvent of the eluate was evaporated as described above.

HPLC conditions

The residues of the extracts were dissolved in 50 μl for serum and bile and in 100 or 200 μl for urine (non-conjugated and enzymically liberated eugenol, respectively) of mobile phase for the HPLC analysis. With the 20-μl sample loop on the Rheodyne valve, 20 μl of each sample were injected and chromatographed under isocratic conditions at room temperature. Elution with a mobile phase at pH 2 [1000 ml of 0.005 *M* potassium hydrogen phosphate and 10 ml of 8.5% (w/v) orthophosphoric acid–acetonitrile–methanol (9:6:1, v/v)] separated the compounds. The eluent flow-rate was 2.5 ml/min. The retention times were 5.5 min for eugenol and 6.8 min for the I.S. Comparison with the retention times of authentic eugenol and I.S., coinjection and mass spectra of collected fractions confirmed their identities.

RESULTS

Extent of enzymic cleavage

The extent of the enzymic cleavage was controlled by eugenol-free samples spiked with the sulphuric acid ester of eugenol. Ten spiked 0.5-ml samples containing 5.158 μg of the ester per ml (corresponding to 3.00 μg of eugenol) were analysed. The eugenol concentration found was 3.0364 ± 0.0964 μg/ml (mean ± S.D.) and the intra-assay coefficient of variation was 3.2%.

Extraction efficiency

The extraction efficiencies for eugenol from serum, urine and bile under the conditions reported are shown in Table I. The recovery was measured at concentrations representative of the ranges of the eugenol levels found in volunteers and patients.

Sensitivity

Using the procedure described, the limit of detection for eugenol was 10 ng/ml in serum, 2 ng/ml in urine and 10 ng/ml in bile (signal-to-noise ratio 5:1). For the values of serum, urine and bile, 0.5-ml drug-free samples spiked with eugenol were extracted.

Calibration

For the preparation of the calibration curves known amounts of eugenol in methanolic solution were added to drug-free serum, urine and bile with a microlitre syringe. Unspiked samples were also extracted and analysed to show the absence of interfering signals. After addition of the I.S. the samples containing different concentrations of eugenol were extracted and analysed as described. The peak-height ratio of eugenol to the I.S. was calculated and plotted against the concentrations of eugenol. All calibration curves were linear in the ranges measured: 0.2–10 μg/ml in serum, 0.02–1.0 and 5–100 μg/ml in urine

TABLE I

EXTRACTION EFFICIENCY

Sample	Eugenol added (μg/ml)	Recovery (%)
Drug-free serum (0.5 ml)	0.5 2.0 7.0	95.6 ± 2.2
Drug-free urine (0.5 ml)	10.0 50.0 100.0	98.4 ± 2.3
Drug-free urine[a] (5.0 ml)	0.05 0.20 0.70	99.1 ± 3.6
Drug-free bile (0.5 ml)	1.0	76.8 ± 0.3

[a]Concentrations typical for non-conjugated eugenol.

for free and conjugated eugenol, respectively, and 0.2–3.0 μg/ml in bile. These ranges were appropriate for the serum, urine and bile levels monitored and for the pharmacokinetic studies. For the extraction from serum the equation of the calibration curve with the eugenol concentrations given above was $y=0.9954x-0.0154$ ($r=0.999\,58$). The equations of the calibration curve for the extraction from urine and bile were $y=0.0416x+0.0065$ ($r=0.999\,97$) and $y=1.1626x+0.0088$ ($r=0.999\,45$), respectively.

Precision

The precision of the overall eugenol assay was determined by analysing ten 0.5-ml drug-free serum, urine and bile samples containing 1 μg/ml, 10 μg/ml and 1 μg/ml eugenol and 200 ng, 5 μg and 200 ng of the I.S., respectively. One sample of each series was injected ten times to determine the precision of the HPLC quantification. For serum and urine, an additional sample each with a higher eugenol concentration was also injected ten times. The results are listed in Table II.

Accuracy

The intra-assay accuracy of the overall eugenol assay was examined with twenty 0.5-ml serum, urine and bile samples each containing eugenol in concentrations representative of the range of serum, urine and bile levels. Two samples were made up for each concentration. The concentrations were calculated with the calibration curve worked up and analysed together with these

TABLE II

INTRA-ASSAY PRECISION

Sample	Eugenol added (μg/ml)	Calculated concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Precision of the overall eugenol assay[a]			
Serum (0.5 ml)	1 0	1.071 ± 0.035	3.3
Urine (0.5 ml)	10.0	10.085 ± 0.408	4 0
Urine[b] (5.0 ml)	0.1	0.097 ± 0.004	4.4
Bile (0.5 ml)	1.0	0.970 ± 0.060	6.2
Precision of the HPLC quantification (within-run precision)[c]			
Serum (0.5 ml)	1.0	1.076 ± 0.006	0.5
	7.0	7.022 ± 0.127	1.8
Urine (0.5 ml)	10.0	9.931 ± 0.063	0.6
	100.0	99.683 ± 0.802	0.8
Urine[b] (5.0 ml)	0.1	0.099 ± 0.001	0.2
	0.7	0.685 ± 0.004	0.6
Bile (0 5 ml)	1.0	0.995 ± 0.014	1.4

[a]Ten identically prepared samples were carried through the whole assay
[b]Typical concentrations for non-conjugated eugenol.
[c]One sample of each concentration indicated was measured ten times.

TABLE III

ACCURACY OF THE ASSAY

Values are eugenol concentrations in μg/ml.

Serum		Urine		Bile	
Added	Found	Added	Found	Added	Found
4.0	4 16	10.0	9.79	0.20	0.198
6.0	6.10	25.0	25.09	0.50	0.457
6.5	6.58	50.0	50.40	1.00	0.053
3.5	3.40	45.0	43.87	0.00	–
0.8	0.81	60.0	58.54	0.60	0 606
2.0	2.05	30.0	30.21	0.35	0.332
0.5	0.52	130.0	129.25	1.30	1.291
5.0	5.09	7.5	7.41	1.00	0.993
1.5	1.40	90.0	89.60	1 60	1.579
8 0	8.14	75.0	75.14	1.50	1.484

series. Table III shows the results of accuracy determinations of twenty 0.5-ml spiked serum, urine and bile samples, respectively.

Selectivity

In this assay, the following constituents of essential oils did not interfere with eugenol; isoeugenol, eugenol methyl ether, eugenol acetate, vanillin, thymol, ferulic acid, caffeic acid, homovanillic acid, anisic acid and homoveratric acid. Also, the eugenol metabolites co-extracted during the work-up did not interfere [16]. The absence of interfering endogenous peaks in serum, urine and bile after the work-up had been determined beforehand.

Applications

A series of serum and urine samples from volunteers and bile samples from patients with an indwelling T-tube was screened by the method to obtain pharmacokinetic data for eugenol in humans. In serum and bile no unconjugated eugenol was detected. In 24-h urine less than 0.1% of the dose was excreted as eugenol. After enzymic cleavage of the serum, urine and bile samples, measurable amounts of eugenol were always liberated. Fig. 2 shows one example

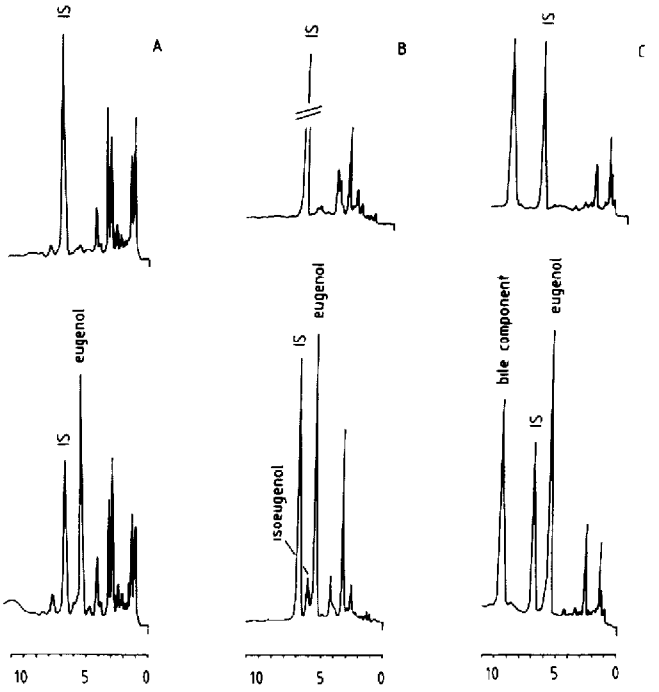


Fig. 2. Chromatograms of extracts of body fluids after enzymic hydrolysis. (A) Serum; (B) urine; (C) bile. Attenuation: 0.02, 0.16 and 0.04 for A, B and C, respectively; the attenuation in the top (drug-free) and bottom (after eugenol ingestion) pairs of traces was always identical.

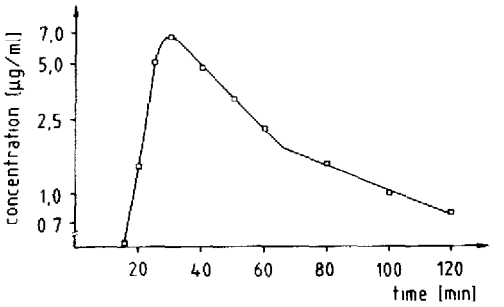


Fig. 3. Pharmacokinetics of eugenol conjugates in serum of a volunteer after oral application of 150 mg of eugenol; free eugenol never appeared in measurable concentrations.

for each body fluid: the top chromatogram is always from the drug-free fluid, the bottom chromatogram was obtained after enzymic hydrolysis. The kinetics of eugenol conjugates were measured for six volunteers, and Fig. 3 shows an example. The concentration of eugenol in 6-h bile was 1.04 ± 0.490 µg/ml (mean ± S.D. from four patients), range 0.33–1.57 µg/ml (corresponding to less than 0.5% of the dose).

DISCUSSION

In contrast to many other constituents of essential oils, eugenol can be analysed by HPLC with UV detection. For eugenol, a phenol with a non-polar side-chain, different organic solvents have been used for extraction [13–15]. In this investigation several solvents (hexane, heptane, chloroform, dichloromethane and diethyl ether) were tested. The best results (with respect to maximal recovery and minimal interferences) were obtained with *n*-hexane, and only for bile samples was further treatment necessary. Removal of the organic solvent was the crucial point of the assay. The time necessary for evaporating the samples to dryness depended on the amount of *n*-hexane used, and it was necessary to keep this time to a minimum otherwise losses of eugenol occurred.

The sensitivity depended on the selection of the wavelength of 220 nm. The UV spectrum of eugenol showed a maximum at 220–230 nm and a smaller one at 278 nm. Eugenol was monitored by different authors [13–15,17,18] at several wavelengths in the range 242–280 nm. The measurement at 220 nm used in this investigation yielded a gain in sensitivity of a factor of ca. 4 compared with that at 270 nm and a factor of 16 compared with that at 254 nm.

The quality control carried out showed the excellent recovery as well as the high precision and accuracy of the HPLC method. The C_{18} HPLC column could be used for all samples measured over a period of more than a year without loss of separation efficacy. The analysis time for each sample was short. No derivatization was required. Thirty-two samples could be worked up and measured

in one working day. Selectivity, sensitivity, reliability and simplicity of handling make this assay suitable for pharmacokinetic studies. An example of a pharmacokinetic profile obtained is shown in Fig. 3.

Attempts to develop a GC assay with flame ionization detection sensitive enough for eugenol extracted from body fluids failed. Such an assay would be well suited to the quantification of eugenol in essential oils, but it was not sensitive and specific enough for quantification in body fluids. Using a mass spectrometer as detector, the required specificity could be achieved [16], but the retention times were twice as long as with HPLC.

ACKNOWLEDGEMENTS

Eugenol, the sulphuric acid ester of eugenol and the eugenol-filled capsules were kindly supplied by M.C.M. Klosterfrau GmbH & Co (Cologne, F.R.G.). We thank Oberarzt Dr. B. Schneider of the Department of Surgery, University of Bonn, Bonn, F.R.G., for making available the bile samples. I.U.F. was supported by the Robert-Bosch-Stiftung (Stuttgart, F.R.G.).

REFERENCES

1 IARC Monogr., 36 (1985) 75.
2 E. Gildemeister and F. Hoffmann, Die Ätherischen Öle, Vol. IIId, Akademie-Verlag Berlin, Berlin, 4th ed., 1960, p. 430.
3 US Food and Drug Administration, Scientific Literature Review of Eugenol and Related Substances in Flavor Usage, Flavor and Extract Manufacturers Association of the United Sates, Washington, DC, 1978, p 1.
4 H. Wagner and L. Sprinkmeyer, Drug Res., 23 (1973) 749.
5 H. Glasl and H. Wagner, Dtsche Apoth. Ztg., 114 (1974) 146.
6 Analytical Methods Committee, Analyst, 106 (1975) 593
7 Analytical Methods Committee, Analyst, 106 (1981) 456.
8 M. Delaforge, P. Janiaud, P. Levi and J.P. Morizot, Xenobiotica, 10 (1980) 737.
9 M.G. Moshonas and P.E Shaw, J. Agric Food Chem., 26 (1978) 1288.
10 E.O. Oswald, L. Fishbein, B.J Corbett and M.P. Walker, J. Chromatogr., 73 (1972) 59.
11 J.A. Boutin, G. Siest, A.M. Batt, E. Solheim and R.R. Scheline, Anal. Biochem., 135 (1983) 201
12 E. Solheim and R.R. Scheline, Xenobiotica, 6 (1976) 137.
13 R.A. Miller, N E. Bursell, C.K. Ricketts and H. Jordi, J. Dent. Res., 58 (1979) 1394.
14 M.S.F. Ross, J. Chromatogr , 160 (1978) 199.
15 Y. Hashimoto, K. Kawanishi, H. Tomita, Y. Uhara and M. Moriyasu, Anal. Lett., 14 (1981) 1525.
16 I.U. Fischer, G.E. von Unruh and H J. Dengler, Xenobiotica, (1990) in press.
17 M.A. El-Sayed, Y.A. Mohamed and N.A. Abdelsalam, Pharmazie, 31 (1976) 361
18 E. Burtscher, H. Binder, R. Conan and O. Bobleter, J. Chromatogr., 252 (1982) 167.