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Determination of methyleugenol in rodent plasma by high-performance liquid chromatography

S.W. Graves*, S. Runyon

Bioanalytical Chemistry, Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201, USA

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Abstract

A method has been developed and validated for the analysis of methyleugenol, a volatile flavoring agent, in rodent plasma at concentrations from 0.050 to 10.0 μg/ml. The method involves the addition of acetonitrile to the plasma, removal of the protein precipitate, and analysis of the supernatant by reversed-phase (C_{18}) HPLC using an acetonitrile–water mixture with UV detection. The precision, accuracy, sensitivity, and specificity of the method were assessed. The stability of methyleugenol in plasma, during freeze–thaw cycles, refrigerated at the extract stage, and during the analysis was evaluated. The recovery of methyleugenol from plasma was also determined. This method was found to be acceptable for plasma concentrations in toxicokinetic studies of methyleugenol in rodents. Over 400 samples from toxicokinetic studies have been successfully analyzed to date. Kinetic data from a preliminary single administration intravenous and oral study in rats is also presented.

1. Introduction

Methyleugenol is used commercially as a fragrance (23 000 kilograms per year) and flavoring agent (4500 to 9000 kilograms per year) since the early 20th century. The FDA approved its use in foods and the Flavor and Extract Manufacturers Association has given it GRAS (generally regarded as safe) status. Methyleugenol is one of a large family of allylphenol derivatives that occur naturally in the volatile oil fraction of various plants. It has a long history of use as a major and active ingredient in traditional medicines. The National Toxicology Program has selected methyleugenol for chronic toxicity testing based on its widespread presence and its structural similarity to safrole and estragole, which are considered weak human carcinogens.

The purpose of this work was to develop and validate a method capable of analyzing rat and mouse plasma samples following intravenous and oral administration of methyleugenol. There is no previously published data on the analysis of methyleugenol itself in plasma or other biological fluids, although there are numerous articles on the chromatographic analysis and quantitation of methyleugenol as a component of natural mixtures (see Refs. [1–4] for recent examples). The metabolism of methyleugenol in Wistar rats following oral and intraperitoneal administration has been established by analysis of urine and bile [5]. Approximately 90% of the dose was found in the urine within 24 h. Metabolism studies of safrole and estragole [6,7] produced similar re-

* Corresponding author.

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sults. The metabolism of eugenol in humans has also been studied [8]. This work involved the analysis of urine, plasma, and bile for eugenol and its conjugates following a single oral dose. The method for the analysis of eugenol itself consisted of extraction with hexane, evaporation of the extract to dryness, and subsequent analysis by reversed-phase HPLC. Initial attempts to use a similar sample preparation scheme were unsuccessful with methyleugenol because of its greater volatility which resulted in significant losses of methyleugenol at low concentrations whenever any solvent evaporation was used. This method eliminates the need for an evaporation step by directly injecting plasma that has been denatured by adding acetonitrile onto the HPLC system.

2. Experimental

2.1. Chemicals and materials

Methyleugenol, FCC grade, was purchased from Elan Chemical Co. (Newark, NJ, USA). 3,4-Dimethoxystyrene, technical grade, and eugenol, 99%, were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Isomethyleugenol, reagent grade, was purchased from Pfaltz and Bauer (Waterbury, CT, USA). Structures for these four allylphenol derivatives are shown in Fig. 1. Acetonitrile, HPLC grade, was purchased from Burdick and Jackson (Muskegon, MI, USA) and used without purification. Deionized water was purified with a Milli-Q (Millipore Corporation, Bedford, MA, USA) water purification system. Rodent plasma was purchased from Taconic Farms (Germantown, NY, USA).

2.2. Calibration standards

Calibration curves were prepared on three separate days in the following manner. Two stock solutions were made by dissolving 1 and 0.5 g of methyleugenol in 100 ml of acetonitrile. These two stocks were further diluted 1 to 100 with acetonitrile producing solutions of 100 and 50 μg/ml. These stocks were used on all days. The 100 μg/ml solution was diluted 1 to 10 and 1 to 50 with acetonitrile giving solutions of 10 and 2 μg/ml. The 50 μg/ml solution was diluted 1 to 10 and 1 to 100 with acetonitrile giving solutions of 5 and 0.5 μg/ml. These standards were prepared daily. Twenty (20) μl of the 100, 50, 10, 5, 2, and 0.5 μg/ml solutions were added to 200 μl of blank plasma to produce the plasma calibration standards. Four replicates were made daily at each concentration.

2.3. Quality control samples

Quality control (QC) samples were prepared just prior to initiation of the method validation at nominal concentrations of 0.25, 1.00, and 5.00 μg/ml by diluting 1 ml of three, independently prepared, acetonitrile solutions, whose concentrations were 2.5, 10.0, and 50.0 μg/ml, to 10 ml with blank plasma. Aliquots (200 μl) of these solutions were then transferred to the tubes used for the sample analysis and stored at −20°C until analyzed.

Plasma obtained from 5 separate animals was analyzed to serve as blanks.

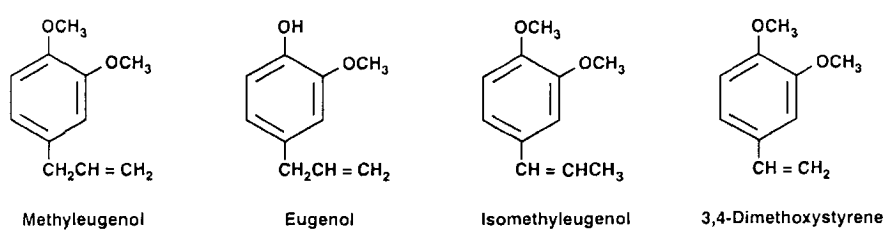

Fig. 1. Structures of methyleugenol and other allylphenol derivatives.

2.4. Experimental design

The validation study was performed on three separate days by two different analysts. Each day of the validation included the preparation and extraction of two calibration curves and the extraction of three sets of QC samples for each calibration curve. In addition, the first day of the validation included the 5 blank plasma samples and solvent standards at concentrations equivalent to the theoretical final concentration of the calibration standards. The second day included assessment of "extract" stability for the time required to inject a typical size run. The third day included the analysis of QC samples which had been taken through 2 freeze–thaw samples and "extracts" which had been stored for 4 days at 5°C. The analysis of the standards, QCs, and other samples was begun as soon as practical following preparation.

2.5. Analysis

The calibration standards and QCs were prepared as described above. They were allowed to warm to room temperature. A 20-μl volume of acetonitrile was added only to the QCs to bring their total volume to the same volume as the calibration standards. The acetonitrile composition of the calibration and QCs prepared, therefore, varied slightly (i.e. 62 vs. 65%). This small difference in acetonitrile composition was shown to have no impact on the analysis. A 300-μl volume of internal standard solution, 3,4-dimethoxystyrene in acetonitrile at a concentration of 2 μg/ml, was added to calibration standards and QCs. Specificity samples were prepared by adding 320 μl of acetonitrile to 200 μl of blank plasma. Solvent standards were made by mixing 200 μl of Milli Q water, 20 μl of the appropriate methyleugenol working stock, and 300 μl of internal standard solution.

The tubes were sealed, vortex-mixed for about 30 s, placed in a −5°C freezer for 5 min, and centrifuged at 500 *g* for 5 min. The supernatant was filtered through a BioRad (Richmond, CA, USA) 0.45-μm Prep-Disc filter into an HPLC vial with a microvial insert.

All samples were analyzed using the HPLC system shown in Table 1.

2.6. Calculations

A weighted $(1/y)$ linear regression curve was calculated relating initial plasma concentration of the standards to their relative chromatographic response (methyleugenol/internal standard) for each of the six standard curves. These standard curves were used to determine the concentrations of the QCs and to back calculate the relative error (residuals) for each point in the standard curve.

Table 1
HPLC system

HPLC instrumentation	Spectra-Physics (Fremont, CA, USA) Model SP880 with a Model SP8875 Autosampler injecting 50 μl from a 200-μl loop in a variable volume mode
Detection	Applied Biosystems (Foster City, CA, USA) Model 757 with a detection wavelength of 230 nm
Column heater	Flatron Model CH-30 set at 40°C
Analytical column	Metachem Technologies (Torrance, CA, USA) Inertsil 5 μm ODS-2, 15.0 × 0.46 cm
Guard column	Metachem Technologies Inertsil 5 μm ODS-2 cartridge
Mobile-phase components	Solvent A: water–acetonitrile (53:47, v/v) Solvent B: acetonitrile
Solvent gradient	Isocratic at 100% A for 20 min, then program to 19% A in 5 min with a final hold of 10 min. The gradient is necessary to remove late-eluting lipophilic materials which interfere with subsequent injections.
Flow-rate	0.7 ml/min

3. Results and discussion

3.1. Calibration

The method exhibited linearity over the range 0.050–10.0 μg/ml with an average coefficient of determination of 0.9999 for the six curves. A typical standard curve is shown in Fig. 2. The column resolution of methyleugenol from the internal standard was 6.0. The column asymmetry for methyleugenol was 1.05. The slopes of the six curves showed excellent agreement with a coefficient of variability of 0.21%. The average *y*-intercept value for the curves was 0.001965. Statistical evaluation of the *y*-intercepts from these six curves showed that only two of the six included the point (0,0) at the 95% confidence level. Nine additional standard curves prepared and analyzed in conjunction with subsequent toxicokinetic studies in rats and mice showed similar results with respect to the *y*-intercept with three of the nine containing the point (0,0) at the 95% confidence level. This data could indicate the presence of a small peak eluting at the same time as methyleugenol which results in the small positive *y*-intercept value, or, because of the small and consistent variance across all concentrations of the standards and the fact that a weighted regression was applied to the data, more likely it is an anomaly related to the high precision and weighted regression. The effect of weighting on the precision and accuracy of calibration is discussed in more detail by Land et al. [9]. Regardless of the cause for the intercept, the method produced acceptable precision and accuracy for all standards and QCs based on recommended criteria [10,11]. Quality control charts of all QC data are presented in Figs. 3–5. Although five of the 45 average concentration values fell outside the ± 2s confidence limits, all individual and average concentrations were well within the method acceptance criteria of ± 15% of target.

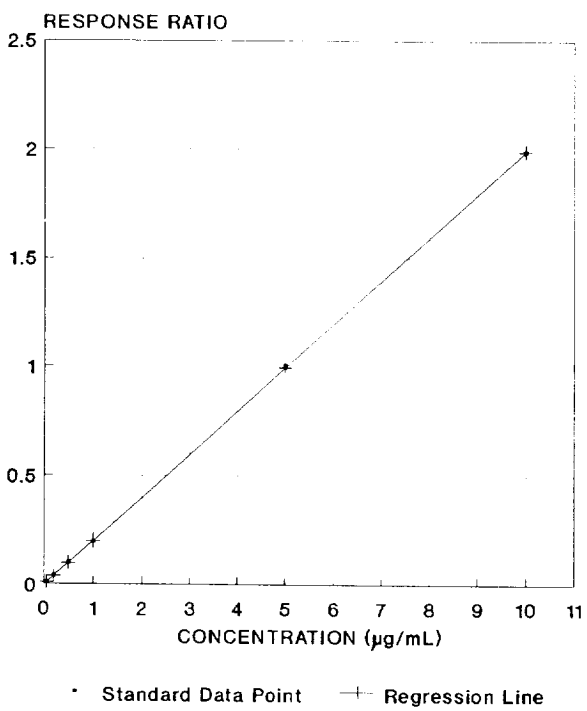

Fig. 2. Typical plasma calibration curve.

3.2. Precision and accuracy

Results from the daily (first day of validation) and day-to-day precision and accuracy for the standards are shown in Table 2.

3.3. Limit of quantitation

The limit of quantitation, defined as the lowest standard with daily and day-to-day relative er-

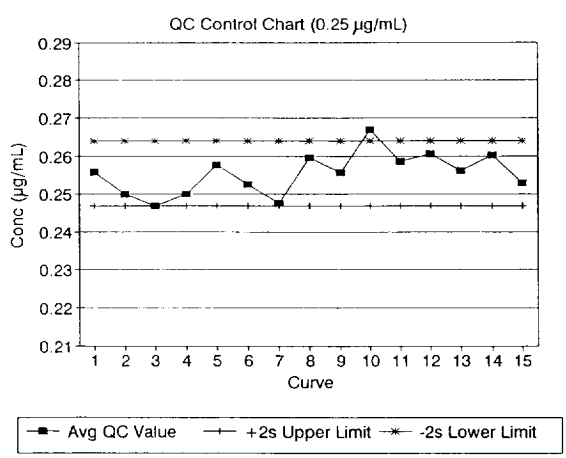

Fig. 3. QC Control Chart (0.25 μg/ml).

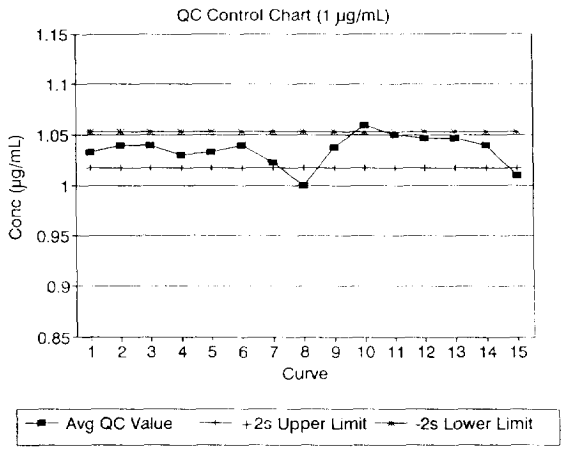

Fig. 4. QC Control Chart (1.0 µg/ml).

rors and relative standard deviations of 20 percent or less, was 0.050 µg/ml.

3.4. Selectivity

No interfering peaks for the methyleugenol or internal standard were seen in chromatograms from five undosed rodents. Chromatograms from a blank, 0.050, and 10.0 µg/ml standards are shown in Fig. 6. Eugenol and isomethyleugenol, potential metabolite/degradation products, were found to be chromatographically resolved from both methyleugenol and 3,4-dimethoxystyrene, the internal standard. All other urinary and biliary metabolites reported by Solheim and

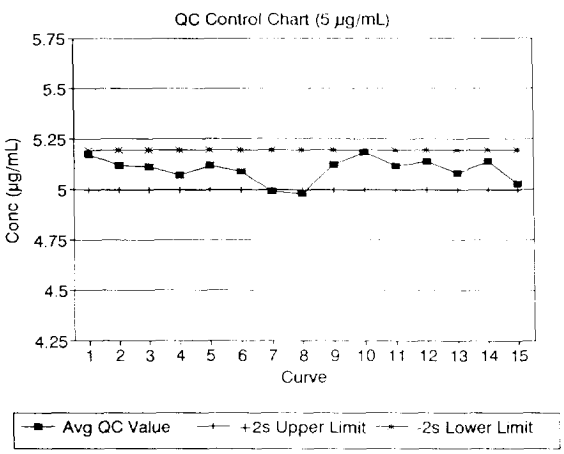

Fig. 5. QC Control Chart (5.0 µg/ml).

Table 2
Standards precision and accuracy

Standard concentration (µg/ml)	Concentration found (mean ± S.D.) (µg/ml)	C.V. (%)	R.E. (%)
Within-day (n = 4)			
0.050	0.048 ± 0.006	12.0	−4.0
0.200	0.206 ± 0.008	3.9	3.0
0.499	0.503 ± 0.008	1.6	0.8
1.00	1.01 ± 0.01	1.1	0.2
4.99	4.98 ± 0.06	1.1	−0.2
10.0	10.0 ± 0.1	1.0	0
Day-to-day (n = 12)			
0.050	0.049 ± 0.006	12.5	−2.0
0.200	0.202 ± 0.011	5.4	1.0
0.499	0.501 ± 0.009	1.8	0.4
1.00	1.01 ± 0.01	1.4	1.0
4.99	4.98 ± 0.04	0.8	−0.2
10.0	10.0 ± 0.0	0.5	0

Scheline [5] were commercially unavailable and could not be evaluated chromatographically. Most of these metabolites are significantly more polar than methyleugenol and would likely be eluted very early in the analysis under the chromatographic conditions employed.

3.5. Recovery

Although extraction was not used in the classical sense, a recovery study was done by comparing four aqueous standards to four plasma standards at the six standard concentrations. Both

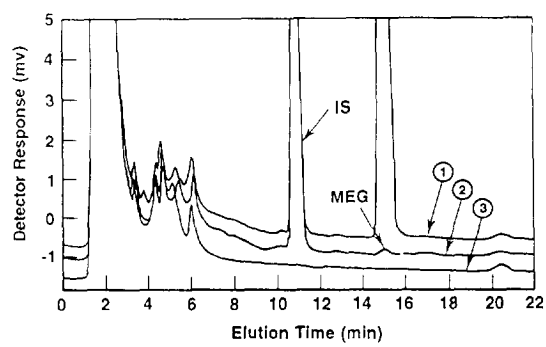

Fig. 6. Chromatograms of blank and spiked rodent plasma. (1) Blank plasma; (2) 0.05 µg/ml plasma standard; (3) 10 µg/ml plasma standard.

sets of standards were processed identically. Recoveries for the methyleugenol were calculated on a point-to-point basis by dividing the peak response of each plasma sample by the average peak response of the four water standards of the same concentration and multiplying by 100. The recovery of the internal standard was calculated for each plasma sample by dividing its response by the average response of all water standards and multiplying by 100. The average recoveries of the standards from the lowest to highest concentrations were 96.4, 130.5, 129.4, 130.5, 134.9, and 130.0. The average recovery of the internal standard was 136.2. We believe the lower relative recovery at 0.050 μg/ml is caused by the evaporation of methyleugenol. The absolute recovery values above 100 are caused by the lower final analysis volume of the plasma standards relative to the aqueous standards. This volume difference occurs when the acetonitrile is added to the plasma causing plasma proteins to precipitate and the supernatant volume is smaller than when no precipitation occurs. A limited scale experiment showed that the final volume of plasma to which acetonitrile was added was approximately 400 μl compared to a final volume of 500 μl for water to which acetonitrile was added. The data indicated that recovery was acceptable and consistent except at the lowest concentration, where it averaged 30% less than the other concentrations. Despite this lower recovery, the low standard had acceptable precision and accuracy. This shows that any attempt to lower the sensitivity of the method would need to look carefully at recoveries at lower concentrations.

3.6. Stability

The stability of methyleugenol at −20°C in plasma, after 2 freeze–thaw cycles, and stored as an extract at 5°C was evaluated using the QC samples. The results are shown in Table 3. The values inside the parentheses are a percentage of the initial value. In addition, a peak purity analysis was done on one QC from each concentration using a waters 996 photodiode-array detector. The resulting UV spectra matched methyleugenol for all three. The purity and threshold angles indicated a homogeneous peak in all cases. The stability of methyleugenol during the analysis was also evaluated by injecting the same set of QC samples at the beginning and end of a run. The values at the beginning were 0.247, 1.04, and 5.11. At the end of the run they were 0.267, 1.04, and 5.07.

3.7. Ruggedness

The validation results from the runs conducted by different analysts using different HPLC instruments with columns of different lots from the same supplier fully met all acceptance criteria. Over 400 mouse and rat samples were subsequently analyzed as part of a single-administration toxicokinetic study in batches consisting of 12 calibration standards, at least 9 QC samples, and approximately 50 plasma samples. No degra-

Table 3
Stability

	Concentration found (μg/ml)		
	0.25 μg/ml	1.0 μg/ml	5.0 μg/ml
Initial values ($n = 6$)	0.256	1.03	5.18
Freezer storage ($n = 6$)	0.242 (94.5)	0.99 (96.1)	5.11 (98.6)
Freeze–thaw ($n = 6$)	0.249 (97.3)	1.01 (98.0)	4.99 (96.1)
Extract ($n = 6$)	0.255 (99.6)	1.00 (97.1)	5.07 (97.8)

Values in parentheses are percentage of the initial value.

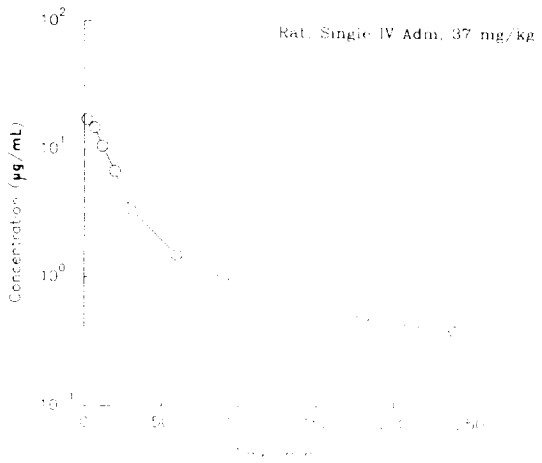

Fig. 7. Plasma concentration–time profile (37 mg/kg i.v.).

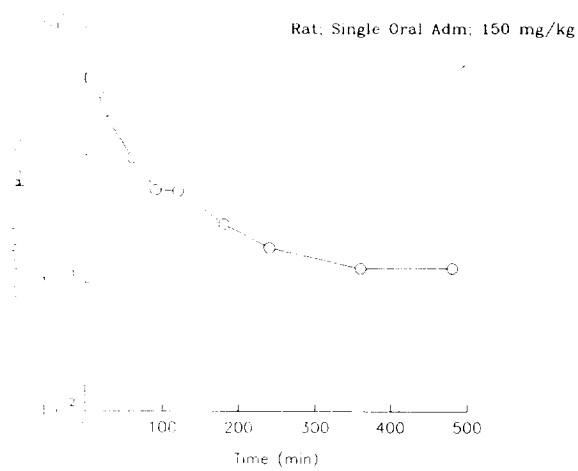

Fig. 9. Plasma concentration–time profile (150 mg/kg oral).

dation in resolution or peak shape was seen over the course of a run. Carryover of endogenous material or methyleugenol from previous injections was not found.

3.8. Toxicokinetic studies

Preliminary and definitive single administration toxicokinetic studies of methyleugenol in rodents have been conducted and the plasma samples analyzed with this method. Figs. 7–9 show the plasma concentration–time profile for rats dosed intravenously at 37 mg/kg and orally at 37 and 150 mg/kg orally in a preliminary range-finding toxicokinetic study. Fig. 10 shows the chromatograms from all the intravenously dosed animals. The AUCs using the trapezoidal method were 470.3, 97.0 and 225.6 μg/ml/min for the i.v. and low and high oral doses respectively. Half lives, $t_{1/2}$, were 30 and 30–60 min for the i.v. and oral doses respectively.

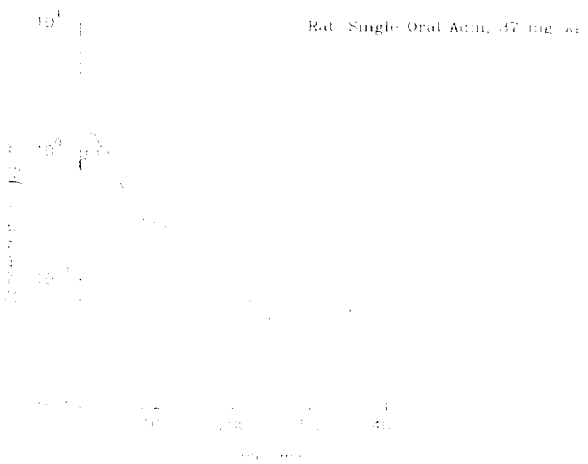

Fig. 8. Plasma concentration–time profile (37 mg/kg oral).

4. Conclusions

The method met all acceptance criteria established during the method development stage to produce acceptable data for toxicokinetic studies in rodents. It was linear over the range 0.050–10.0 μg/ml with reproducible slopes and acceptable y-intercepts. The precision and accuracy, evaluated using the relative errors and coefficients of variability of the calibration standards and QC samples, were less than 15 percent at the lowest calibration concentration and less than 10 percent at all other concentrations. Blank rodent plasma did not contain any endogenous materials, which interfered with the chromatography, and available standards of potential metabolites or degradants were found to be chromatographically resolved from methyleugenol. The recovery of the methyleugenol and internal stan-

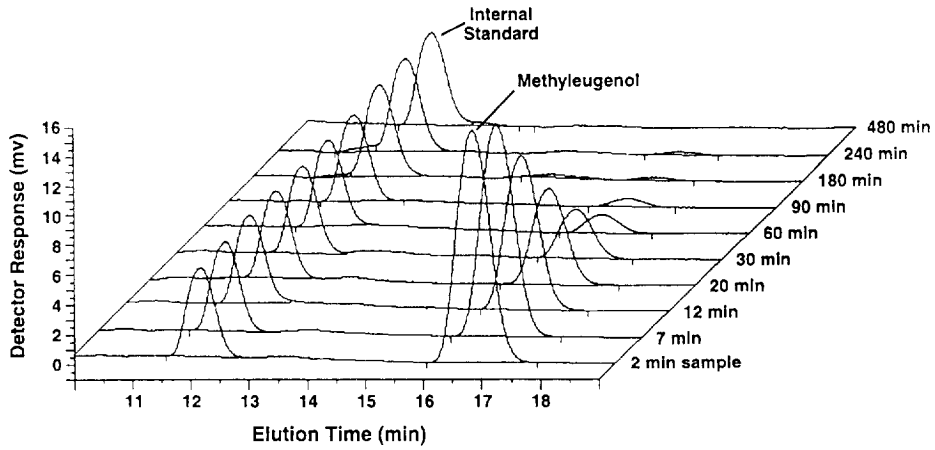

Fig. 10. Chromatograms from i.v. dosed rats.

dard was acceptable at all concentrations; however the recovery of the lowest standard was approximately 30% lower than at the other concentrations; perhaps due to the volatility of methyleugenol. Plasma samples were stable for at least 34 days when stored at −20°C and through two freeze–thaw cycles. Peak purity assessment of the methyleugenol peak in stored QCs by photodiode-array analysis found the peaks to be homogeneous with the UV spectrum of the peak matching that of methyleugenol. Extracts were stable for at least four days at 5°C storage and during a full chromatographic analysis cycle. The method has been used successfully to analyze samples from a series of toxicokinetic studies.

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