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Journal of Chromatography B, 726 (1999) 303–307

JOURNAL OF CHROMATOGRAPHY B

Short communication

High-performance liquid chromatographic method for determination of vanillin and vanillic acid in human plasma, red blood cells and urine

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Received 27 August 1998; received in revised form 14 December 1998; accepted 21 December 1998

Abstract

A simple high-performance liquid chromatographic method was developed for the determination of vanillin and its vanillic acid metabolite in human plasma, red blood cells and urine. The mobile phase consisted of aqueous acetic acid (1%, v/v)–acetonitrile (85:15, v/v), pH 2.9 and was used with an octadecylsilane analytical column and ultraviolet absorbance detection. The plasma method demonstrated linearity from 2 to 100 μg/ml and the urine method was linear from 2 to 40 μg/ml. The method had a detection limit of 1 μg/ml for vanillin and vanillic acid using 5 μl of prepared plasma, red blood cells or urine. The method was utilized in a study evaluating the pharmacokinetic and pharmacodynamic effects of vanillin in patients undergoing treatment for sickle cell anemia.

Keywords: Vanillin; Vanillic acid

1. Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde; 4HM) is typically used as a flavoring agent in confectionery, beverages, pharmaceuticals, foods and perfumery. Vanillin is also hypothesized to induce an antisickling effect through covalent bonding of the aldehyde group of the vanillin molecule with the hemoglobin group in the red blood cell [1]. The metabolite vanillic acid (4-hydroxy-3-methoxybenzoic acid) is not known to be pharmacologically active in the red blood cells. There have been no published high-performance liquid chromatography (HPLC) or gas chromatography (GC) methods to date for the measurement of vanillin and vanillic acid in human plasma, red blood cells and urine. The method detailed in the present communication uti-

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00031-6

lizes a simple sample preparation step for each matrix and does not require the use of an internal standard. In addition, this method employs current HPLC midbore column technology which provided sufficient sensitivity and reduced mobile phase requirements and subsequent waste disposal.

2. Experimental

2.1. Chemicals

Vanillin (CAS 121-33-5) and vanillic acid (CAS 121-34-6) were purchased from Sigma (St. Louis, MO, USA). Glacial acetic acid was HPLC-grade and acetonitrile was Optima HPLC-grade and both were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure distilled and deionized water was prepared in-laboratory and filtered prior to use.

2.2. HPLC equipment and mobile phase

The HPLC equipment consisted of a Varian pump Model 9010 solvent delivery system (Walnut Creek, CA, USA). The analytical column was a Spherex octadecylsilane (ODS), 150 mm×3.2 mm I.D., 3 μm packing (Phenomenex, Torrance, CA, USA). The C_{18} guard column, 30 mm×4.6 mm I.D., 40–50 μm pellicular packing (Alltech, Deerfield, IL, USA) was replaced prior to each analytical run which typically consisted of approximately 50 samples. For plasma and red blood cell analysis, the mobile phase consisted of aqueous acetic acid (1%, v/v)–acetonitrile (85:15, v/v), pH 2.9. For urine analysis, the mobile phase gradient (linear) consisted of aqueous acetic acid (1%, v/v)–acetonitrile with composition and time as follows (90:10, v/v for 10 min; 70:30, v/v at 13 min and hold 5 min). The mobile phase was degassed daily using helium sparging and used at a flow-rate of 0.50 ml/min for plasma and 0.60 ml/min for urine and red blood samples. Typical operating pressure was 13.6 MPa at ambient temperature. An injection volume of 5 μl of the prepared sample was accomplished using a WISP Model 712 (Waters, Milford, MA, USA) autosampler. Component detection was achieved using a Shimadzu SPD-6A UV detector (Tokyo, Japan) set at 280 nm. The detector operated at high sensitivity setting with a 1 s response time. A 345 kPa back-pressure regulator (SSI, State College, PA, USA) was coupled to the detector outlet to prevent outgassing. Data acquisition and component computations were performed using Turbochrom (PE Nelson, Norwalk, CT, USA) chromatography software on a Hewlett-Packard (Palo Alto, CA, USA) 486 DX-33 personal computer.

2.3. Standard and control preparation

Stock standards of vanillin and vanillic acid (1 mg/ml) were prepared in methanol and stored at 4°C. Working plasma standards of 2, 5, 10, 25, 50 and 100 μg/ml vanillin and vanillic acid were prepared using blank human plasma as the diluent. Working urine standards of 2, 5, 10, 20 and 40 μg/ml vanillin and vanillic acid were prepared using deionized water as the diluent. Control plasma samples of 3, 17.5 and 75 μg/ml and control urine samples of 3, 15 and 30 μg/ml were spiked with vanillin and vanillic acid and were prepared using blank human plasma and deionized water as the diluent, respectively. All working standards and controls were stored and maintained at −20°C with the patient samples.

2.4. Sample conditions

Institution Review Board approved informed consent was obtained from the study volunteers. Prior to dosing the fasting volunteer, a heparin lock was placed in an arm vein and baseline studies obtained. The volunteer was then given a capsule containing 5, 10, 20 or 40 mg/kg vanillin along with 240 ml of water. Blood samples were drawn from the heparin lock at precisely timed intervals into heparin coated tubes after removal of 5 ml of blood to clear the line. Blood samples were centrifuged at 1600 *g* for 15 min. Plasma was removed and the buffy coat discarded. Both plasma and red cell pellets were stored at −20°C in polypropylene tubes. Urine samples were collected at predetermined time points with 10-ml aliquots stored at −20°C in polypropylene vials. Prior to analysis, plasma samples were thawed to ambient temperature, mixed thoroughly by inversion and centrifuged at 800 *g* for 5 min to eliminate fibrinous material. Red blood cell samples were

thawed to ambient temperature and mixed thoroughly by inversion. Urine samples were thawed to ambient temperature, mixed thoroughly by inversion, and allowed to stand for 15 min for particulates to settle out.

2.5. Sample preparation

Plasma samples were prepared by pipetting 150 μl of plasma and 150 μl acetonitrile into a polypropylene bullet centrifuge tube. Plasma proteins were precipitated by vortex mixing for 15 s. The samples were centrifuged at 13 000 *g* for 10 min. The clear supernatant was transferred to polypropylene autosampler microvials. Red blood cell lysis was completed by pipetting 100 μl of red blood cells and 100 μl deionized water into a polypropylene bullet centrifuge tube. The contents were vortex mixed for 15 s and sonicated for 1 min. A 200-μl volume of acetonitrile was added and the proteins were precipitated by vortex mixing for 15 s. The samples were centrifuged at 13 000 *g* for 10 min. The supernatants were filtered through a 0.22-μm nylon Acrodisc into polypropylene autosampler microvials. Urine samples were prepared by pipetting 40 μl of urine and 460 μl of deionized water into a 12×75 mm polypropylene culture tube and vortex mixing for 10 s. The diluted urine samples were transferred to polypropylene autosampler microvials. For plasma, red blood cell and urine preparations, 5 μl was injected into the HPLC system.

3. Results and discussion

3.1. Chromatography

The method demonstrated excellent chromatographic selectivity with no endogenous plasma interferences at the retention times for vanillic acid and vanillin (5.9 and 9.3 min, respectively) (Fig. 1A). Chromatograms of prepared blank human plasma containing low (2 μg/ml) and high (100 μg/ml) concentrations of vanillic acid and vanillin (Fig. 1B and C, respectively) indicated good detector response and baseline resolution from endogenous substances with an analytical run time of 13 min. A typical chromatogram for plasma from one subject dosed with vanillin is shown in Fig. 1D. Chromatograms demonstrating the selectivity and sensitivity of the method for urine and red blood cell samples are shown in Figs. 2 and 3, respectively. To extend column lifetime, the analytical ODS column was flushed after each analytical run (approximately 50 samples) for 2 h at 0.60 ml/min with acetonitrile–deionized water (85:15, v/v) to eliminate retained non-polar substances from the column.

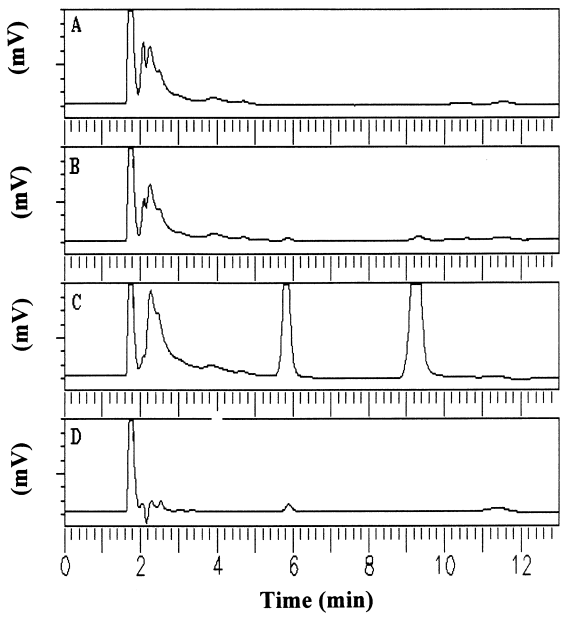

Fig. 1. Chromatograms of (A) prepared blank human plasma, (B) prepared blank human plasma spiked with 2 μg/ml vanillic acid and vanillin, (C) prepared blank human plasma spiked with 100 μg/ml vanillic acid and vanillin, (D) subject plasma sample taken at 20 min. Retention time of vanillic acid (5.9 min) and vanillin (9.3 min).

3.2. Linearity, limit of detection and computations

The plasma method was linear throughout the tested concentration range of 2 to 100 μg/ml with a mean correlation coefficient (n=13 analytical runs) of 0.9998 and 0.9997 for vanillic acid and vanillin, respectively. The urine method was linear throughout the concentration range of 2 to 40 μg/ml with a mean correlation coefficient (n=8 analytical runs) of 0.9993 and 0.9990 for vanillic acid and vanillin,

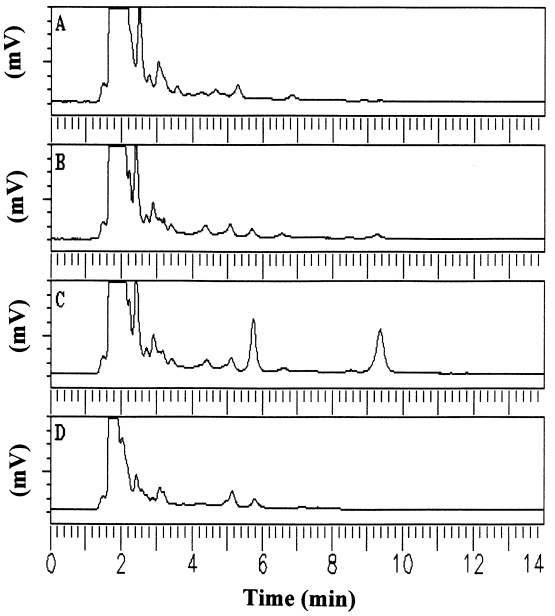

Fig. 2. Chromatograms of (A) prepared blank human urine, (B) prepared blank human urine spiked with 5 μg/ml vanillic acid and vanillin, (C) prepared blank human urine spiked with 40 μg/ml vanillic acid and vanillin, (D) subject urine sample representing 4–8 h. Retention time of vanillic acid (5.7 min) and vanillin (9.3 min).

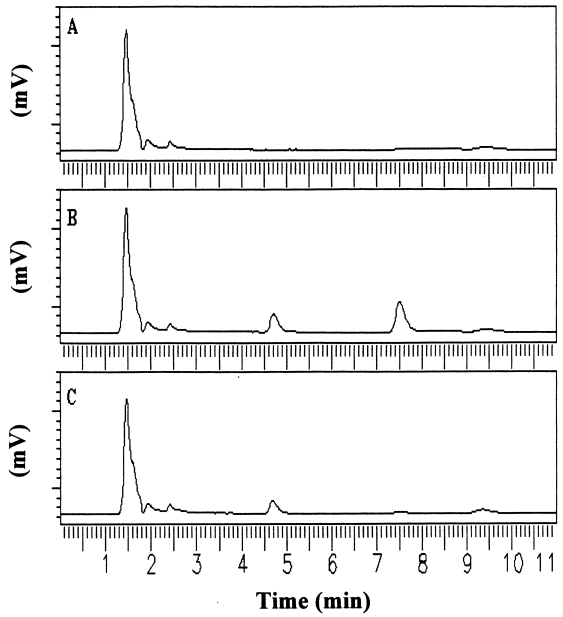

Fig. 3. Chromatograms of (A) prepared blank red blood cells, (B) prepared blank red blood cells spiked with 20 μg/ml vanillic acid and vanillin, (C) subject red blood cells sample taken at 30 min. Retention time of vanillic acid (4.7 min) and vanillin (7.5 min).

respectively. The limit of detection for the method (1 μg/ml) was determined by evaluation of a spiked standard in each matrix at 1 μg/ml (n=3). For plasma, red blood cells and urine component calculations, normal linear regression utilizing external standardization and peak height was used for vanillic acid and vanillin. The lowest standard calibrator for plasma, red blood cells and urine was used as the limit of quantitation for reporting calculated patient results.

3.3. Accuracy, precision and recovery

The accuracy and precision for the method was determined by evaluation of replicate control samples over the course of all analytical runs at vanillic acid and vanillin concentrations of 3, 17.5 and 75 μg/ml for plasma and 3, 15 and 30 μg/ml for urine. The accuracy of the method was reported as the error of theoretical versus measured vanillic acid and vanillin concentrations and was 7.5, −4.8 and −4.9% for vanillic acid plasma control samples and 8.6, −2.4 and −2.9% for vanillin plasma control samples (n=31). The error was −7.7, −1.2 and 3.8% for vanillic acid urine control samples and −4.0, −7.3 and 1.7% for vanillin urine control samples (n=16). The precision of the method was reported as relative standard deviation (RSD) and was 12.4, 4.1 and 4.5% for vanillic acid plasma control samples and 10.6, 3.2 and 4.8% for vanillin plasma control samples (n=31). The RSD was 17.5, 5.9 and 5.9% for vanillic acid urine control samples and 13.4, 6.4 and 5.8% for vanillin urine control samples (n=16). Absolute recovery for the plasma method was evaluated by comparing standards which were prepared in blank plasma versus standards prepared in deionized water. The average absolute recovery for the plasma and red blood cell method was determined to be 99%. Absolute recovery for the urine method was not performed as the method does not employ formal extractions (e.g., liquid–liquid, solid-phase). In addition, the standards and controls

used for analysis were treated identical to the patient plasma and urine samples thus controlling for potential errors in micropipetting.

4. Conclusions

A simple method was developed for evaluating vanillin and its metabolite, vanillic acid in plasma, red blood cells and urine. We employed a simple sample preparation for each matrix and eliminated the need for time-consuming extractions and internal standards, thus making this method cost effective. In addition, this method utilized current midbore HPLC column technology which provided sufficient selectivity, sensitivity and reduced mobile phase requirements, saving both procurement and subsequent waste disposal cost. The use of the midbore column did not necessitate modifications to the HPLC system (i.e., injection volume and detector cell volume). The method was employed in the evaluation of plasma, red blood cells and urine samples from a pharmacokinetic and pharmacodynamic study of vanillin in sickle cell patients without significant methodological problems.

Acknowledgements

This study was supported by the National Institute of Health Grant No. IND35096 and the FDA Orphan Drug Program Grant No. FD-R-00948.

References

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