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A validated LC method for the determination of vesamicol enantiomers in human plasma using vancomycin chiral stationary phase and solid phase extraction

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Abstract

An enantioseparation high performance liquid chromatographic (HPLC) method was developed and validated to determine D-(+)- and L-(−)-vesamicol in human plasma. The assay involved the use of a solid phase extraction for plasma sample clean up prior to HPLC analysis utilizing a C18 Bond-Elute column. Chromatographic resolution of the vesamicol enantiomers was performed on a vancomycin macrocyclic antibiotic chiral stationary phase (CSP) known as Chirobiotic V with a polar ionic mobile phase (PIM) consisting of methanol:glacial acetic acid:triethylamine (100:0.1:0.05 (v/v/v)) at a flow rate of 1.0 ml/min and UV detection set at 262 nm. All analyses were conducted at ambient temperature. The method was validated over the range of 1–20 μg/ml for each enantiomer concentration ($R^2 > 0.999$). Recoveries for D-(+)- and L-(−)-vesamicol enantiomers were in the ranges of 96–105% at 3–16 μg/ml level. The method proved to be precise (within-run precision ranged from 1.3 to 2.7% and between-run precision ranged from 1.5 to 3.4%) and accurate (within-run accuracies ranged from 0.8 to 3.4% and between-run accuracies ranged from 1.7 to 5.0%). The limit of quantitation (LOQ) and limit of detection (LOD) for each enantiomer in human plasma were 1.0 and 0.5 μg/ml ($S/N = 3$), respectively.

Keywords: Enantioseparation; Vesamicol; Vancomycin chiral stationary phase; Human plasma

1. Introduction

It has recently been established that frequently only one of the two enantiomers of a drug is pharmacologically active, whereas the other can be inactive or toxic [1]. Differences between the biological activity of enantiomers arise because of differences between protein binding and transport, mechanism of action, rate of metabolism, rate of clearance, and persistence in the environment [2–4]. Despite this, many compounds such as drugs, agrochemicals, and food additives have been marketed as racemic mixture.

On the other hand, the enantioselective character of pharmacokinetic processes leads to different plasma concentration–time profiles for the constituent enantiomers [5]. For this reason, the evaluation of the disposition of a drug employed as a racemic mixture based on data achieved from the non-selective assay is

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not only seriously limited, but can also be highly misleading, particularly when attempting to relate plasma concentration to a pharmacological effect or therapeutic benefit [6]. This is true for both newly developed drugs and agents with long-term use in clinical practice, but now subject to reevaluation in this area. The investigation of enantioselectivity represents a great challenge for clinical pharmacology in terms of the control of individual variability of clinical responses. The clinical relevance of the phenomenon is particularly important for drugs of low therapeutic index administered as racemates.

A prerequisite for progress in acquiring knowledge of stereospecific pharmacodynamics and pharmacokinetics of optical isomers is the development of an enantioselective analytical methodology. In the last two decades extensive research has been performed on the resolution of the enantiomer by liquid chromatography (LC) and capillary electrophoresis (CE) because of their high efficiency, speed, preparative capability, wide range of applications, and reproducibility. A search of the literature indicates that the most interesting research in this area involves the development of new chiral selector and different types of chiral selector have been used in chromatography for direct enantiomers resolution (without derivatization) [1].

Macrocyclic antibiotics have been introduced by Armstrong et al as powerful chiral selector in liquid chromatography [7], thin-layer chromatography [8], and capillary electrophoresis [9]. The glycopeptides macrocyclic antibiotic such as vancomycin has been widely used as a chiral stationary phase (CSP) and a great variety of racemic compounds have been resolved on it [10]. The enantioselectivity of these chiral selector due to several reasons: (i) they are amphoteric (i.e. contain acidic and basic ionizable groups); (ii) they have the necessary geometry and functionalities that accentuate chiral recognition in solution; and (iii) they contain both hydrophilic and hydrophobic moieties [11].

A simplified approach has proven very effective for the resolution of a broad spectrum of racemate analytes. The first consideration in this direction is the structure of the analytes. If the compound has more than one functional group capable of interacting with the stationary phase and at least one of those groups is on or near the stereogenic center, then the first mobile phase choice would be the polar organic phase. Due to the strong polar groups present in the macrocyclic peptides, it was possible to convert the original mobile phase concept to 100% methanol with an acid/base added to effect selectivity. The key factor in obtaining complete resolution is still the ratio of acid to base [12]. The importance and superiority of macrocyclic antibiotics as chiral selector, in comparison with other chiral selector, is because they can be used in normal and reversed phases with greater stability and capacity [1].

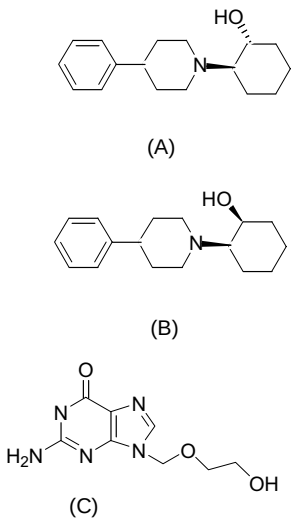

Fig. 1. The chemical structure of (A) L-(−)-vesamicol, (B) D-(+)-vesamicol, and (C) acyclovir (IS).

Vesamicol, 2-(4-phenylpiperidino) cyclohexanol (Fig. 1) is a potent synthetic inhibitor of vesicular acetylcholine storage. Vesamicol inhibits the transport of acetylcholine into synaptic vesicles in cholinergic nerve terminals [13,14]. Pharmacological studies showed that the inhibition of acetycholine transport is stereospecific and concentration-dependent between the two enantiomers. L-(−)-Vesamicol is 25-fold more potent than D-(+)-vesamicol [15]. A chiral high performance liquid chromatographic (HPLC) separation was reported using a Chiralcel OD column without quantitative studies [16]. Recently, Zhou and Stewart [17] determined vesamicol enantiomers in human serum by capillary electrophoresis using sulfated-β-cyclodextrin.

This method is not first method for vesamicol chiral assay. However, it provides an alternative way for the

assay of vesamicol enantiomers. This study describes a validated HPLC method for the quantitation of L-(−)- and D-(+)-vesamicol enantiomers in human plasma using vancomycin CSP column commercially known as Chirobiotic V as the chiral selector.

2. Experimental

2.1. HPLC system

The analysis was performed by a 1525 Binary HPLC pump, 717 plus utosampler, 2487 Dual λ absorbance detector and In-line degasser AF, (Water Milford, MA, USA). The column used in this study was the macrocyclic antibiotic vancomycin known as Chirobiotic V (25 cm × 4.6 mm i.d.) purchased from Advanced Separation Technologies (Whippany, NJ, USA). The mobile phase was methanol:glacial acetic acid:triethylamine (100:0.1:0.05 (v/v/v)). The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon, Millipore (Yonezawa, Japan). The flow rate was 1.0 ml/min the sample injection volume was 20 μl and the chromatograms were monitored by UV detection at a wavelength of 262 nm.

2.2. Chemicals and reagents

(±)-Vesamicol, D-(+)-vesamicol, L-(−)-vesamicol, and acyclovir (internal standard) were purchased from RBI (Natick, MA, USA). HPLC-grade methanol and triethylamine were obtained from Fisher Scientific (Fairlawn, NJ, USA). Analytical reagent grade glacial acetic acid was purchased from BDH Chemicals (Poole, UK). Deionizes water was purified using a cartridge system (Picotech Water System, RTP, NC). Water Oasis HLB and Sep-Pak C18, C8, and CN cartridges were obtained from Water Corp (Milford, MA). Human plasma was obtained from the King Faisal Specialist Hospital Blood Bank (Riyadh, KSA). All plasma samples were stored at −20 ± 5 °C.

2.3. Preparation of stock and standard solution

Stock solutions of individual D-(+)-vesamicol, L-(−)-vesamicol, and acyclovir (internal standard) (1 mg/ml) were prepared in methanol. A seven-point non-zero calibration standard curve, ranging from 1 to 20 ug/ml, was prepared by spiking the drug free plasma with appropriate volume of D-(+)- and L-(−)-vesamicol. The quality control (QC) samples, at three concentration levels, i.e. 3, 9, and 16 μg/ml were prepared in similar manner from the stock solution. Before the spiking, the drug-free plasma was tested to make sure that there was no endogenous interference at retention times of D-(+)-vesamicol, L-(−)-vesamicol, and internal standard. The quality control samples were extracted with the calibration standards to verify the integrity of the method.

2.4. Preparation of plasma samples

Human plasma sample (0.5 ml) was placed into 1.5 ml Eppendrof tube and accurately measured aliquots of D-(+)- and L-(−)-vesamicol were added. Then 20 μl of the internal standard was added to each tube and diluted with water to 1 ml and vortex vigorously for 60 s to give final concentration of 3, 9, and 16 μg/ml of each vesamicol enamtiomers. Waters Oasis HLB and Sep-Pak C18, C8 and CN cartridges were studied. Cartridges were conditioned with 2 × 1 ml methanol and 2 × *l* ml deionized water before applying the plasma samples. Care was taken that the cartridges did not run dry. The entire spiked plasma samples were then transferred to solid phase extraction (SPE) cartridges. Vacuums were then applied to obtain a flow through the cartridges of 0.5 ml/min. The cartridges were then washed with 2 × 500 μl deionized water. The cartridges were then dried under vacuum for 5 min. All SPE cartridges were eluted with 2 × 500 μl methanol containing 1% TEA. The eluting solvent was evaporated to dryness using a Savant speed vac concentrator (Farmingdale, NJ, USA). The residue was dissolved in 500 μl deionized water and 20 μl was injected into an HPLC system.

2.5. Selectivity

The selectivity of the assay was checked by analyzing four independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with vesamicol enantiomers and internal standard.

2.6. Linearity

Calibration plots for the D-(+)- and L-(−)-vesamicol enantiomers in plasma were prepared by diluting stock solutions with pooled human plasma to yield seven concentrations over the range of 1–20 μg/ml for each enantiomer, respectively. Calibration standards at each concentration were extracted and analyzed in triplicate. Calibration curves of vesamicol enantiomers were constructed using the observed analyte peak area versus nominal concentrations of the analytes. Least squares linear regression analysis of the data gave slope, intercept, and correlation coefficient data. From this data, a first order polymonial model was selected for each analyte.

2.7. Precision and accuracy

The within-run and between-run accuracy and precision of the assays in plasma were determined by assaying three quality control samples in triplicate over a period of 3 days. The concentrations represented the entire range of the calibration curves. The lowest level was at third the expected limit of quantitation (LOQ) for each enantiomer. The second level was at the mid-point of the calibration curves and the third level was at 80% of the upper concentration of the calibration curves. Calibration curves were prepared and analyzed daily and linear models were used to determine concentrations in the quality control samples. The nine measured concentrations per concentration level (triplicates from three runs) were subjected to estimate the within-run and between-run precision. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Precision was reported as % relative standard deviation (%R.S.D.).

2.8. Limit of detection and limit of quantitation

The limit of detection (LOD) and the limit of quantitation were determined as 3 and 10 times the baseline noise, respectively, following the United States Pharmacopoeia [18]. The results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear squares treatment of the results along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of the residuals ($S_{y/x}$). The good linearity of the calibration graphs and the negligible scatter of experimental points are clearly evident by the values of the correlation coefficient and standard deviation [19]. The robustness of the method is demonstrated by the versatility of the experimental factors that affect the peak area.

2.9. Recovery

The absolute recoveries of each enantiomer from plasma was calculated by comparing drug peak area of the spiked analyte samples to unextracted analyte of stock solution that has been injected directly into an HPLC system. The assay absolute recovery for each compound, at each concentration, was computed using the following equation: absolute recovery = (peak area of extract)/(mean peak area of direct injection) × 100.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The chemical structures of D-(+)- and L-(−)-vesamicol, and acyclovir (IS) are shown in Fig. 1. Vancomycin chiral stationary phase has been widely used for enantiomer resolution because it very effectively recognizes the enantiomers of anionic compounds. The selectivity towards these compounds is because of the presence of amine groups in the chiral selector and, in fact, better recognition is obtained at acidic buffer pH below or close to the isoelectric point of the antibiotic. The polar ionic mode (PIM) has been described as a novel method to obtain difficult enantioselective separation with macrocyclic antibiotic-based chiral stationary phases [20]. This approach uses a non-aqueous polar component (methanol) with glacial acetic acid or trifluoroacetic acid and an amine such as triethylamine which are necessary to achieve enantioseparation. In this study, baseline separation of the vesamicol enantiomers was achieved on the vancomycin CSP column with a polar ionic mobile phase consisting of methanol:glacial acetic acid:triethylamine

Table 1
Chromatographic parameter data for vesamicol enantiomers and internal standard in spiked human plasma

Analyte	T_R (min) (mean ± S.D., $n = 3$)	k (mean ± S.D., $n = 3$)	Tailing factor[a]	α[b]	Rs[c]
Acyclovir	5.82 ± 0.05	5.08 ± 0.04	1.0	[d]	[d]
L-(−)-Vesamicol	11.48 ± 0.03	10.83 ± 0.07	1.0	2.13	7.66
D-(+)-Vesamicol	13.22 ± 0.02	12.66 ± 0.06	1.1	1.17	2.00

[a] Calculated at 5% peak height.
[b] Separation factor, calculated as k_2/k_1.
[c] $Rs = 2(t_2 - t_1)/(w_{b2} + w_{b1})$, where t_2 and t_1 are the retention of the second and first peaks and w_{b2} and w_{b1} are the half peak width of the second and first peaks.
[d] Not calculated.

(100:0.1:0.05 (v/v/v)) (Table 1). No enantiomeric separation were observed in the absence of triethylamine when the mobile phase consisted of methanol:acetic acid (100:0.1 (v/v)). This could be explained on the basis of strong repulsive effects between the protonated amino groups of the analyte molecules and of the CSP. An increase of the triethylamine concentration in the mobile phase (to about 0.2%) decreases the retention factors of the studied analytes. This demonstrates that it is the concentration (ratio) of acetic acid and triethylamine in mobile phase that has a substantial influence on the retention factors and not the ionic strength of the mobile phase which was constant.

Aboul-Enein and Ali [21] reviewed the possible bonding between the enantiomers and the macrocyclic glycopeptide antibiotics CSPs. The most important bondings involved are π–π complexation, hydrogen bonding, inclusion complexation, dipole interactions, steric interactions, anionic and cationic bindings. These bonding are a result of the complex structures of this CSP which consists of sugar moieties, phenyl rings, along with several chiral centers, inclusion baskets, hydrogen donor and acceptor sites. It has been reported that these bonding sites are responsible for the surprising chiral selectivities of these antibiotics [7]. The studied enantiomers of vesamicol (Fig. 1) contain nitrogen and oxygen atoms, along with benzene ring, which interact with the complimentary groups on the chiral selectors (antibiotics). The inclusion baskets and the other functional moieties provide the chiral sites in which the enantiomers fit stereogenically in different fashion which result in the chiral discrimination between the vesamicol enantiomers. Besides, the steric effect is also playing an important role for the chiral resolution of the studied drug on these CSPs.

3.2. Applications to spiked human plasma

In the course of developing a solid phase extraction procedure for plasma sample clean up, several types of cartridges were investigated (Water Oasis HLB and Sep-Pak C18, C8, and cyanopropyl). The cyanopropyl cartridge showed interference endogenous plasma peaks at retention time of the analytes. An octyl (C8) SPE column was also found to be unacceptable due to low recoveries (<60%) for vesamicol enantiomers and internal standard. Oasis HLB cartridge showed recoveries in excess 70% whereas an octadecyl (C18) SPE column gave high recoveries for vesamicol enantiomers and internal standard (more than 90%) while at the same time removing endogenous interference. Figs. 2 and 3 show chromatograms of a blank plasma sample and a calibration sample, respectively.

3.3. Linearity

The linear regression analysis of D-(+)-vesamicol, L-(−)-vesamicol enantiomers was constructed by plotting the peak area ratio of each enantiomer to the internal standard (y) versus analyte concentration (μg/ml) in spiked plasma samples (x). The calibration curves were linear in the range of 1–20 μg/ml for D-(+)- and L-(−)-vesamicol, with correlation coefficient (R^2) of more than 0.999 (Table 3). A typical calibration curve has the regression equation of $y = 0.0053x + 0.0586$ for D-(+)-vesamicol and $y = 0.0059x - 0.0578$ for L-(−)-vesamicol.

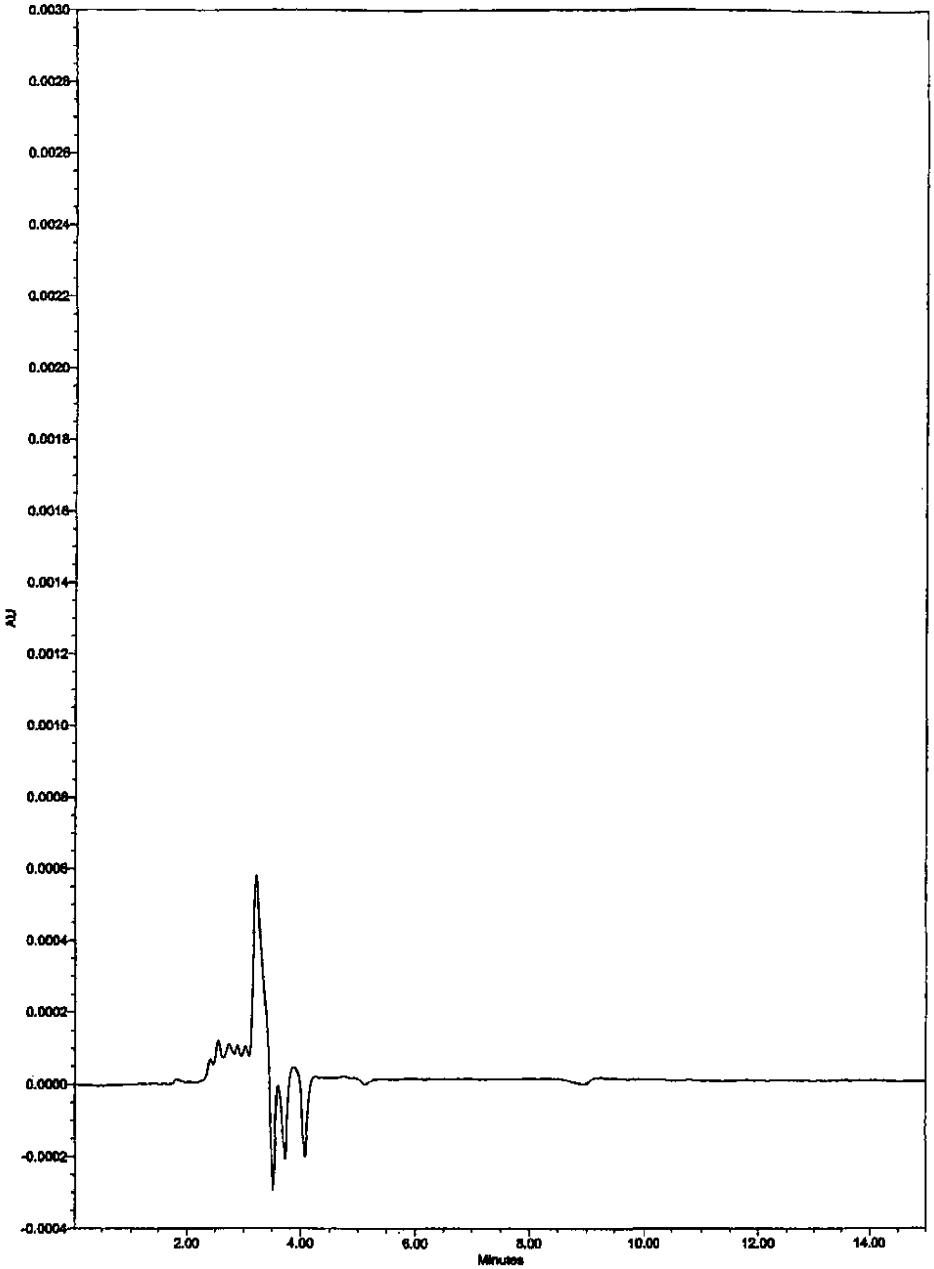

Fig. 2. Chromatogram of blank pooled human plasma.

3.4. Precision and accuracy

A summary of the accuracy and precision results is given in Table 2. The acceptance criteria (within-run and between-run %R.S.D. of <15% and an accuracy between 85 and 115%) were met in all cases. The precision and accuracy of the method were determined by using plasma samples spiked at three levels (Table 2). The data indicate that within-run precision and accuracy ($n = 3$) as expressed by %R.S.D. and

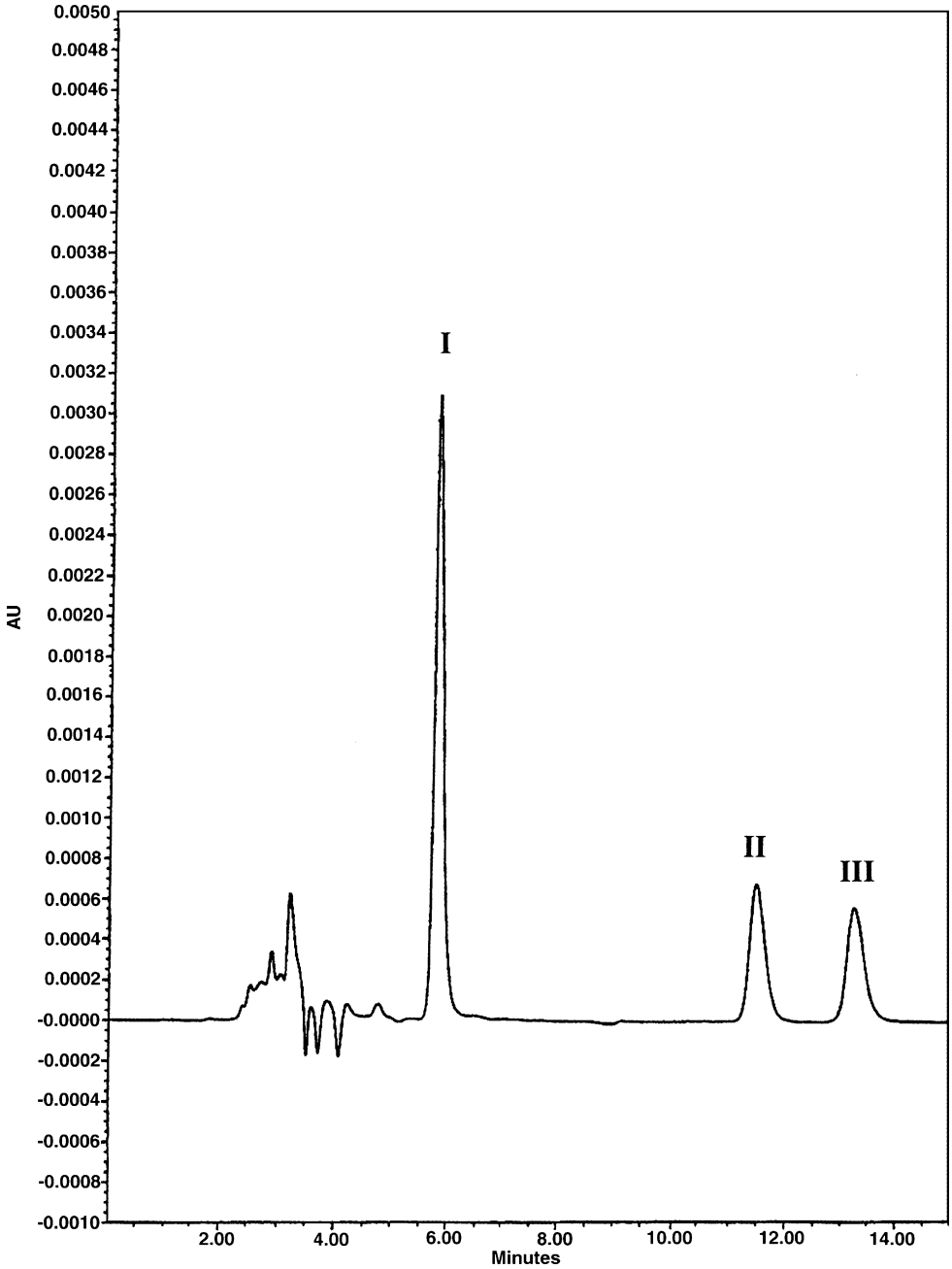

Fig. 3. Chromatogram of pooled human plasma spiked with 20 μg/ml acyclovir [I], 5 μg/ml L-(−)-vesamicol [II], and 5 μg/ml D-(+)-vesamicol [III].

percentage error were 1.3–2.7 and 0.8–2.8%, respectively for D-(+)-vesamicol and 1.5–2.4 and 1.7–3.4% for L-(−)-vesamicol, respectively. The between-run precision and accuracy ($n = 9$) expressed by %R.S.D. and percentage error were 1.5–2.2 and 1.7–4.1% for 2.5–3.4% and 2.7–5.0% for D-(+)-vesamicol and L-(−)-vesamicol respectively. The detailed analytical data are shown in Table 2.

Table 2
Accuracy and precision of human plasma spiked with vesamicol enantiomers

Analyte	Nominal concentration (μg/ml)	Measured concentration (μg/ml)	Error (%)	R.S.D. (%)
Within-run[a]				
D-(+)-Vesamicol	3	2.95 ± 0.08	1.7	2.7
	9	8.93 ± 0.12	0.8	1.3
	16	15.56 ± 0.26	2.8	1.7
L-(−)-Vesamicol	3	2.94 ± 0.07	2.0	2.4
	9	8.85 ± 0.13	1.7	1.5
	16	15.45 ± 0.32	3.4	2.1
Between-run[b]				
D-(+)-Vesamicol	3	3.08 ± 0.09	2.7	2.9
	9	9.36 ± 0.32	4.0	3.4
	16	16.80 ± 0.42	5.0	2.5
L-(−)-Vesamicol	3	3.05 ± 0.06	1.7	1.9
	9	9.32 ± 0.14	3.6	1.5
	16	16.66 ± 0.37	4.1	2.2

[a] Mean ± S.D. based on $n = 3$.
[b] Mean ± S.D. based on $n = 9$.

3.5. Limit of detection and limit of quantitation

The LOD as defined in Section 2 were 1.0 μg/ml for D-(+)- and L-(−)-vesamicol enantiomers (Table 3). The LOQ of each calibration graph was 0.5 μg/ml for each enantiomer. Table 3 also shows the results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the least squares treatment of the results along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of the residuals ($S_{y/x}$).

Table 3
Validation parameters for the determination of vesamicol enantiomers using the proposed method

Parameters	D-(+)-Vesamicol	L-(−)-Vesamicol
Concentration range (μg/ml)	1–20	1–20
Intercept (a)	−0.0205	−0.0981
Slope (b)	0.6449	0.6582
Correlation coefficient (R^2)	0.9995	0.9996
$S_{y/x}$	0.0374	0.0468
S_a	0.0029	0.0038
S_b	0.0002	0.0003
LOQ (μg/ml)	1.0	1.0
LOD (μg/ml)[a]	0.5	0.5

[a] $S/N = 3$.

3.6. Selectivity

The analytical figures of merit for this method are shown in Table 1. D-(+)- and L-(−)-vesamicol enantiomers were well separated under the HPLC conditions applied. Retention times were 11.48 and 13.22 L-(+)- and D-(−)-vesamicol enantiomers, respectively. No interference was observed in drug free human plasma samples. Figs. 2 and 3 show chromatograms of a blank plasma sample and a calibration sample, respectively.

4. Conclusion

A high-performance liquid chromatography method for the determination of D-(+)- and L-(−)-vesamicol in human plasma was developed and validated. The vesamicol enantiomers were separated with a vancomycin chiral stationary phase. The method used an efficient solid phase extraction procedure for sample clean up of plasma. The total run time for this method is 15 min, which allows processing of over 90 samples per day. This method has provided good sensitivity and excellent precision and reproducibility.

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References

[1] H.Y. Aboul-Enein, L.I. Abou-Basha, Chirality and drug hazards, in: H.Y. Aboul-Enein, I.W. Wainer (Eds.), The Impact of Stereochemistry on Drug Development and Use, Wiley, New York, 1997, pp. 1–19 (Chapter 1).
[2] M. Simonyi, I. Fitos, J. Vizy, TIPS 7 (1986) 112–116.
[3] E.P.A. Lehmann, TIPS 7 (1986) 281–285.
[4] T. Walle, K.W. Walle, TIPS 7 (1986) 155–158.
[5] J.S. Oleksiak, J. Bojarski, H.Y. Aboul-Enein, Chirality 14 (2002) 417–435.
[6] M. Islam, J. Mahdi, I. Bowen, Drug Saf. 17 (1997) 149–165.
[7] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, I.R. Chen, Anal. Chem. 66 (1994) 1473–1484.
[8] D.W. Armstrong, Y. Zhou, J. Liq. Chromatogr. 17 (1994) 1695–1707.
[9] D.W. Armstrong, K. Rundlett, G.G. Reid, Anal. Chem. 66 (1994) 1690–1695.
[10] Ph. Duret, A. Foucault, R. Margraff, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 295–312.
[11] K.H. Ekborgott, Y. Liu, D.W. Armstrong, Chirality 10 (1998) 434–483.
[12] H.Y. Aboul-Enein, I. Ali, Il Farmaco 57 (2002) 513–529.
[13] C. Prior, I.G. Marshall, S.M. Parsons, Gen. Pharmacol. 23 (1992) 1017–1022.
[14] I.G. Marshall, S.M. Parsons, Trends Neurosci. 10 (1988) 174–177.
[15] G.A. Rogers, S.M. Parsons, D.C. Anderson, L.M. Nilsson, B.A. Bahr, W.D. Kornreich, R. Kaufman, R.S. Jacobs, B. Kirtman, J. Med. Chem. 32 (1989) 1217–1230.
[16] D.L. Gildersleeve, Y.W. Jung, D.M. Wieland, J. Chromatogr. A 667 (1994) 183–189.
[17] M. Zhou, J.T. Stewart, J. Pharm. Biomed. Anal. 30 (2002) 443–449.
[18] The United State Pharmacopeia, 24th ed., United States Pharmacopeial Convention, Rockville, MD, 2000, p. 2150.
[19] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, Wiley, New York, 1984.
[20] K.M. Fried, P. Koch, I.W. Wainer, Chirality 10 (1998) 484–489.
[21] H.Y. Aboul-Enein, I. Ali, Chromatographia 52 (2000) 679–691.