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Short communication

Method for the simultaneous determination of losartan and its major metabolite, EXP-3174, in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry

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Abstract

A liquid chromatography–electrospray ionization tandem mass spectrometric method was developed for the simultaneous determination of losartan and its major active metabolite, EXP-3174, in human plasma. The two analytes and the internal standard (DuP-167) were extracted from plasma under acidic conditions by using solid-phase extraction cartridges containing a sorbent of copolymer, poly(divinylbenzene-co-*N*-vinylpyrrolidone). The analytes were separated by LC equipped with a reversed-phase C_{18} column, and introduced into the mass spectrometer via the electrospray ion source with pneumatically-assisted nebulization. For LC–MS–MS samples, an isocratic mobile phase consisting of [0.1% triethylamine–0.1% acetic acid (pH 7.1)]–acetonitorile (65:35, v/v) was used, and the assay was monitored for the negative fragment ions of the analytes. The method demonstrated linearity from 1 to 1000 ng/ml for both losartan and EXP-3174. The limit of quantification for both compounds in plasma was 1 ng/ml. This assay method may be useful for the measurement of levels of the two compounds in clinical studies of losartan.

Keywords: Losartan; EXP-3174

1. Introduction

The renin-angiotensin system (RAS) plays a critical role in blood pressure regulation and electrolyte homeostasis. The RAS consists of a cascade of enzymatic reactions leading to the formation of angiotensin (Ang) II, which is a powerful vasoconstrictor [1,2]. Losartan potassium, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[{2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl}methyl] imidazole, monopotassium salt (Fig. 1), is a non-peptide, orally active, highly selective Ang II type I receptor antagonist indicated for the treatment of hypertension with heart failure or renal impairment [3–9]. The major metabolite of losartan, EXP-3174 (Fig. 1), is pharmacologically active [3–6]. To investigate the pharmacokinetic properties of losartan and E-3174 in man, simultaneous assay methods using high-performance liquid chromatography (HPLC) have been developed and validated [3–6]. However, these methods have some analytical disadvantages: low sensitivity, need for large plasma samples, complicated preparation on sampling and long run time for analysis. The assay method developed in our study solved the above

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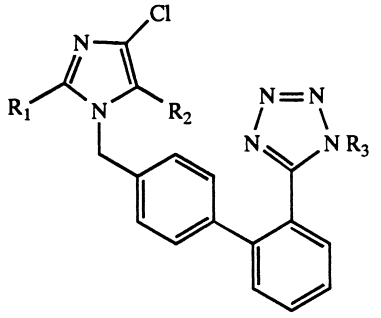


	R_1	R_2	R_3
Losartan potassium	n-C_4H_9	CH_2OH	K
EXP-3174 hydrochloride	n-C_4H_9	COOH	H·HCl
DuP-167	n-C_3H_7	CHO	H

Fig. 1. Structures of losartan potassium, EXP-3174 hydrochloride and the internal standard (DuP-167).

problems by using LC–tandem mass spectrometry (MS–MS) and automatic solid-phase extraction (SPE) in the preparation of LC–MS–MS samples. This is the first report of an LC–MS–MS method for the simultaneous quantification of losartan and EXP-3174 in human plasma. We believe that this assay method utilizing LC–MS–MS features will be useful in the further development of losartan, especially in the case of administering other drugs concomitantly with losartan.

2. Experimental

2.1. Chemicals

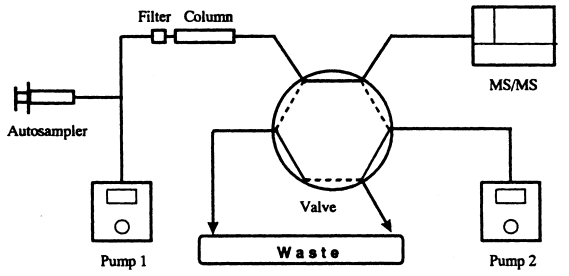
Losartan, EXP-3174 and DuP-167 were obtained from Merck Research Labs. (Rahway, NJ, USA). The structures of losartan, EXP-3174 and the internal standard (DuP-167) used in the assay are shown in Fig. 1. HPLC-grade methanol, ethanol and acetonitrile were purchased from Wako (Osaka, Japan). Guaranteed grade 90% formic acid and triethylamine (TEA), 85% phosphoric acid and acetic acid were purchased from Wako, Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, USA), respectively.

2.2. Instrumentation

A Rapid Trace SPE Workstation and a Turbo Vap LV from Zymark (Hopkington, MA, USA) were used as the automatic equipment for SPE and evaporation in the preparation steps, respectively. A HP 1100 Series from Hewlett-Packard (Waldbronn, Germany) was used as the HPLC system. A Model API 300 triple quadrupole mass spectrometer from Perkin-Elmer Sciex (Concord, Canada) was connected to the HPLC system as an MS–MS detector. A Capcellpak C_{18} (UG120 150×1.5 mm I.D., 5 μm) from Shiseido (Tokyo, Japan) was used for HPLC separation.

2.3. LC–MS–MS conditions

The LC–MS–MS system is shown in Fig. 2. The mobile phase consisted of 650 ml of 0.1% TEA (pH 7.1, adjusted by 0.1% acetic acid) and 350 ml of acetonitrile. Two pumps connected to same isocratic mobile phase were set to a flow-rate of 150 μl/min. The six-port valve was operated according to the time program in Fig. 2. The analytes in extracted samples were separated by the analytical column at 40°C. The analytes were introduced to the mass spectrometer via the electorospray source under atmospheric pressure. The detection was performed by monitoring the negative ions of the parent and



Time (min)	Valve position S : Solid line D : Dotted line
Initial	D
0 - 1.8	D
1.8 - 6	S
6	D

Fig. 2. LC–MS–MS system and the time program of the system.

product compounds combined in selected reaction monitoring mode. The m/z values of the parent and daughter ions were set to 421.2 and 126.7 for losartan, 435.5 and 157.1 for EXP-3174 and 405.0 and 171.0 for DuP-167, respectively. The nebulizer probe temperature was 450°C. N_2 gas was used as the nebulizer and collision gas. The collision energies for the analysis of losartan, EXP-3174 and DuP-167 were set to 32 eV, 29 eV and 32 eV, respectively. Analysis time was 6 min in this assay method.

2.4. Standard preparation

Standard stock solutions of losartan and EXP-3174 were prepared as 1 mg/ml of free form in ethanol solutions. DuP-167 was prepared as 0.2 mg/ml of free form in ethanol solutions. All subsequent dilutions were made with 40% ethanol including 0.1% formic acid. The working standard solutions were a mixture of losartan and EXP-3174. The concentrations were 2, 10, 50, 100, 500, 1000 and 2000 ng/ml for both the intact form and the metabolite. The appropriate internal standard was diluted to a working concentration of 200 ng/ml. These working solutions were stored below −70°C until use.

2.5. Sample preparation

After a 0.4-ml aliquot of plasma was acidified with 0.2 ml of 1 *M* phosphoric acid, 0.2 ml of 200 ng/ml DuP-167 in 40% ethanol including 0.1% formic acid was added to the plasma. The sample was applied to an the Oasis HLB (30 mg/1 ml) extraction cartridge from Waters (Milford, MA, USA), which was conditioned by passing through 2 ml of methanol and 2 ml of water. The cartridge was washed with 1 ml of water and then 3 ml of 30% methanol. Then, the analytes were eluted from the cartridge with 2 ml of methanol. These extraction processes were operated by a Rapid Trace SPE Workstation. After the eluted solution was evaporated at 40°C under N_2 gas, the residual solution was dissolved with 0.2 ml of 40% ethanol including 0.1% formic acid and was sonicated for 3 min. The residual solution was vortexed, and a 40-μl aliquot was injected onto LC–MS–MS system for analysis.

2.6. Quantification

Calibration standards were prepared by adding known amounts of losartan (0.4–400 ng), EXP-3174 (0.4–400 ng) and 40 ng of internal standard to 0.4 ml of control human plasma. The sample extraction and LC–MS–MS analyses were carried out as described above; every analysis with a single standard sample at each concentration. Concentrations of losartan and EXP-3174 were calculated from the linear least-squares fitted line weighted ($1/y^2$) peak-area ratios of losartan and EXP-3174 to the internal standard versus standard concentrations.

3. Results and discussion

3.1. Sample preparation and LC–MS–MS conditions

Since the analytes are weak acids (the pK_a values of the acidic nitrogen in the tetrazole ring are 5.6 and 5.4 for losartan and EXP-3174, respectively; the pK_a value for the carboxy group in EXP-3174 is 4.2) [6], almost all of them exist as nonionic forms in solution at a low pH (≤3.0). In this assay, the sample applied onto the SPE cartridge was acidified to less than pH

3.0 with 1 *M* phosphoric acid. In the state of nonionic forms, the strong binding of the analytes to the copolymer of the SPE cartridge enables sufficient clean-up. However, the analytes were easily eluted by 2 ml of methanol. The evaporated samples were dissolved and acidified by 40% ethanol including 0.1% formic acid.

The analytes ionized by the ESI method were analyzed with negative ion detection because of the efficiency of ionization of analytes. The ESI method had more than 20-fold higher sensitivity than that of the atmospheric pressure chemical ionization (APCI) method. In general, negative ion detection is selective and highly sensitive to compounds with high electron affinity. Losartan, EXP-3174 and DuP-167 have high electron affinity. Therefore, it was considered that these analytes released the acidic proton in the tetrazole ring in a mobile phase of pH 7.1 could get a minus electric charge easily by ESI.

Furthermore, preventing the introduction of plasma-components into the mass spectrometer by utilizing the switching-valve might decrease pollution and increase the number of assay samples.

3.2. Selectivity

Representative chromatograms obtained from control human plasma and plasma spiked mixture of losartan, EXP-3174 and Dup-167 are shown in Fig. 3. There was no interference peak for endogenous components in the chromatograms obtained from control plasma of the six individuals at the peak

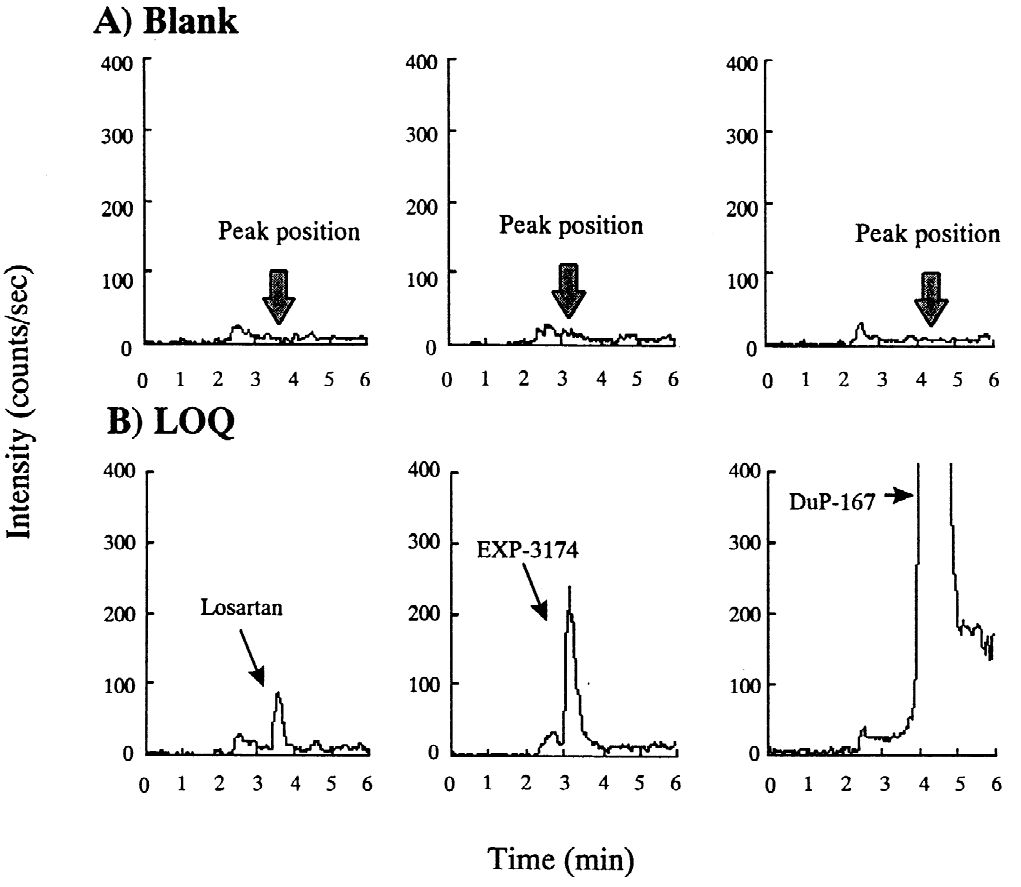


Fig. 3. Typical chromatograms of drug-free plasma (A) and plasma containing 1 ng/ml of analytes (B) for losartan, EXP-3174 and the internal standard (DuP-167).

positions for the analytes. Therefore, it is expected that the assay for clinical samples would be not prevented by the interference peak in this method. Under the chromatographic conditions described, the retention times of losartan, EXP-3174 and internal standard were 3.6, 3.1 and 4.3 min, respectively.

3.3. Linearity

The calibration curves were linear over the range 1 to 1000 ng/ml with mean correlation coefficients ($n=6$ analytical runs) of 0.9970 and 0.9983 for losartan and EXP-3174, respectively.

3.4. Precision and accuracy

Both the intra- and inter-day accuracy and precision of the method were determined by replicate analysis ($n=5$) of control human plasma spiked with losartan and EXP-3174 at all concentrations utilized for constructing the calibration curves. The accuracy and the precision of the method were described as a percentage error of theoretical versus measured concentrations and the percentage of the relative standard deviation (RSD), respectively. Accuracy and precision were calculated at each concentration. The intra-day accuracy was 92.0–102.6% for losartan in the range 1 to 1000 ng/ml and 92.8–107.3% for EXP-3174 in the range 1 to 1000 ng/ml. The intra-day precision was ≤8.6% at all concentrations for losartan, and ≤7.5% in the range 5 to 1000 ng/ml and 12.8% at 1 ng/ml for EXP-3174. The inter-day accuracy was 92.0–106.3% for losartan in the range 1 to 1000 ng/ml and 96.9–103.8% for EXP-3174 in the range 1 to 1000 ng/ml. The inter-day precision was ≤9.2% in the range 5 to 1000 ng/ml and 17.5% at 1 ng/ml for losartan, and ≤7.6% in the range 5 to 1000 ng/ml and 10.6% at the limit of the quantification (LOQ) concentration for EXP-3174 (Fig. 3). The LOQ for both compounds in plasma was 1 ng/ml.

3.5. Recovery

Recovery was determined by comparing peak areas from unextracted standards with those of extracted standards. The mean absolute recoveries of the analytes in the range 1 to 1000 ng/ml from plasma were 64.7–80.5% for losartan and 52.2–70.2% for EXP-3174. Though the recoveries for losartan and EXP-3174 were not constant in the range, the change of the recoveries was not depending on the concentrations of them. The absolute recovery of 40 ng, actual additional quantity in this method, of Dup-167 was 38.7–47.8% over all concentrations of losartan and EXP-3174 for calibration curve. These recoveries of the analytes seem to be low. On the elution-step in SPE, increasing the volume of elute-solvent, the recoveries for losartan and EXP-3174 was unchanged. Therefore, it was suggested that the cause of low recoveries was depression of ionization by plasma endogenous compounds rather than the binding of the analytes to SPE sorbent. However, it was considered that there was no problem in this assay method because the assay method was validated by intra- and inter-day assays.

3.6. Stability

Analysis of standard stability was performed at concentrations of 10 and 1000 ng/ml. After storage for 44 days at −70°C, more than 97.5% of losartan and more than 94.0% of EXP-3174 remained, based on their peak-areas at each concentration. This suggests that the analytes in standard solution were stable for at least 44 days when stored at −70°C.

The stability of losartan and EXP-3174 in an autosampler (4°C), at room temperature and in an evaporator (40°C) was investigated at concentrations of 5 and 500 ng/ml in plasma. LC–MS–MS samples, the samples before application onto the SPE cartridge, and the residue after evaporation were used in the stability studies in the autosampler, at room temperature and in the evaporator, respectively. Losartan and EXP-3174 in extracts from plasma were stable in the autosampler for 60 h at 4°C. After storage for 6 h at room temperature and for 4 h in the evaporator, more than 95.4% of losartan and more than 94.1% of EXP-3174 remained.

We confirmed that repeated freezing and thawing (five cycles) of plasma samples spiked with losartan and EXP-3174 at concentrations of 5 and 500 ng/ml, did not effect the stability of the analytes.

With respect to the stability of both compounds in human plasma, Furtek and Loo reported that the

analytes were stable for 30 weeks when stored at −20°C [6].

The above results indicate that losartan and EXP-3174 are stable enough to be analyzed using this assay method.

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