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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 177–183
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

HPLC—radiometric determination of quinlukast in biological fluids

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Received 29 October 2003; received in revised form 13 January 2004; accepted 14 January 2004

Abstract

An analytical method for the analysis of a novel antiasthmatic drug quinlukast and its metabolites in the plasma, bile and urine was developed. For the analysis, the solid phase extraction method and the C_8 RP-HPLC with radiometric detection of the drug were used. This method enables a quantitative determination of the agent and all of its metabolites (even of those with an unknown structure) in a biological system. The procedure is, therefore, suitable both for the pharmacokinetic analysis of quinlukast and the determination of its elimination pathways.

Keywords: Quinlukast; HPLC; SPE; Metabolites

1. Introduction

Quinlukast (Q), 4-(4-(quinoline-2′-yl-methoxy) phenylsulphanyl)benzoic acid (VUFB 19363, Leciva, Prague, the Czech Republic) is a new potential antiasthmatic drug belonging to the same group as montelukast [1], zafirlukast [2] and pranlukast [3]. Their antileukotrienic effects are comparable. Q is in a preclinical development at present [4]. Leukotrienes (LT) play an important role in pathological processes of inflammation. LT are formed as the products of arachidonic acid biotransformation [5,6]. Compounds influencing the biosynthesis of LT and antagonizing their biological functions are intensively investigated. Q is characterized by inhibition of LT biosynthesis combined with a high affinity to LT receptors and a low toxicity including the absence of an ulcerogenic effect [4]. The chemical structure of Q is shown in Fig. 1. It is a lipophilic compound, probably undergoing an intensive metabolism and elimination in the form of polar metabolites. The incubation of this compound in a rat microsomal fraction and in a primary culture of rat hepatocytes showed that the main metabolic pathway of Q was liver metabolism, especially oxidation of the sulfide bridge to its S-oxide [7], which is usually the principle metabolite in the first stage of biotransformation of such drugs. The other biotransformation reactions probably involve the formation of polar conjugates of Q and its S-oxide and/or formation of further metabolites, e.g., the formation of two dihydrodiol derivates, identified only by means of mass spectrometry [8]. Unfortunately, these substances have not been synthesized as standards so far.

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doi:10.1016/j.jpba.2004.01.014

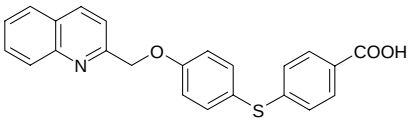

Fig. 1. Chemical structure of quinlukast ($M_w = 387$).

Only the determination of the parent drug in serum, using the SPE-SIA method, has been described in the first journalistic publication concerning Q, which has appeared recently [9].

For that reason a sensitive analytical assay for the pharmacokinetic analysis and for the determination of Q and its metabolites in a variety of biological fluids has to be developed. A procedure for the separation and determination of Q and its metabolites with the employment of SPE-HPLC analysis has been optimized in this study.

The radiolabelled compound was used at our experiments assuming the fact that the chromatographic separation with the radioactive detection allows to determine both the parent drug and its metabolites in the complex biological matrix and providing that endogenous substances do not interfere with the determination of agent under study [10–12]. Q was radiolabelled with tritium (^{3}H) by an isotopic exchange reaction.

2. Experimental

2.1. Reagents and materials

Methanol and acetonitrile (Sigma, St. Louis, USA) were HLPC grade; the other chemicals were of analytical reagent. Universal LSC cocktail, dimethylsulfoxide, Na_2HPO_4, KH_2PO_4 (Sigma) and trihydrogenphosphoric acid (Lachema, Brno, the Czech Republic) were used. The extraction columns Discovery DSC-18LT (55 μm, 100 mg, 1 ml cartridge) were supplied by Supelco (Bellefonte, USA). The water was purified by reverse osmosis (Millipore system, Molsheim, France). Standards of non-radiolabelled Q and its S-oxide were provided by VUFB (Leciva, Prague, the Czech Republic).

^{3}H-Radiolabelled Q was prepared in the Nuclear Research Centre (Rez, the Czech Republic). Its specific activity was 2.8 MBq/mg; radiochemical purity was >95%. Therefore this substance was pre-purified and concentrated before its administration. The purifications were performed repeatedly on the HPLC system under the same conditions as described below. Only the peak of Q was collected. Q dissolved in the mobile phase, obtained during several purifications, was neutralized (pH 7.0) with 0.5 M NaOH and evaporated under a stream of nitrogen.

For perfusion experiments the evaporation residue (purified Q) was re-dissolved in 60 μl of dimethylsulfoxide, then 10 ml of perfusion medium was added as a solubilizer. For in vivo studies the evaporation residue was re-dissolved in 50 μl of dimethylsulfoxide, then 0.6 ml of blank plasma was added as a solubilizer.

2.2. Biological samples

For in vivo study male Wistar rats weighing 240 g were used. In short, the bile duct of anaesthetized rat (Pentobarbital) was cannulated [13]. Q in a dose of 0.36 mg/kg was administered into the Saphena vein in a volume of 0.6 ml. A cannula was inserted into the carotid artery and the blood samples (200 μl) were repeatedly withdrawn to heparinized PE vials and centrifuged at 17,000 × g for 60 s. The bile samples were collected during 30 min periods. The experiments were performed for 120 min after drug administration. A sample of the urine was also taken directly from the urinary bladder at the end of the experiment.

As another example of biological fluids, the samples of perfusion medium and bile from the in situ perfused rat liver preparation were employed [13]. In short, after pentobarbital anesthesia, the bile duct and the portal vein of the rat on a temperature-controlled plate were cannulated. After vena cava cannulation, a recirculating perfusion medium was infused into the hepatic portal vein. After the equilibration period, the agent under study was added to a reservoir (containing 150 ml of perfusion medium) in a volume of 10 ml and in a dose of 120 μg. Input and outflow perfusate samples were repeatedly removed at 10 min intervals in the middle of 10 min periods of bile collection and were centrifuged at 17,000 × g for 10 s and the supernatant was decanted. The experiments were performed for 95 min after drug loading.

2.3. Sample preparation

DSC-18LT SPE column equipped with a needle (0.9 mm × 40 mm) was conditioned with 0.5 ml of

100% methanol, 0.5 ml of 5% methanol and 0.5 ml of 9 mM phosphate buffer (pH 4.9). After the equilibration of cartridge, the biological sample (90 μl of the plasma or the centrifuged perfusion medium, with 9 μl of 100 mM phosphate buffer pH 4.9) was loaded and allowed to flow by gravity. After introducing the sample into the column, it was washed with 1 ml of 9 mM phosphate buffer (pH 4.9) and 0.1 ml of water. Finally, the analyte of interest was eluted from the column with 0.6 ml of 100% methanol and evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 36 μl of dimethylsulfoxide and 84 μl of water. A 100 μl aliquot of the solution was injected into the HPLC system. The samples of the bile and urine were analyzed without pre-separation.

2.4. High performance liquid chromatography

The Agilent Technologies 1100 Series HPLC system (Waldbronn, Germany) was employed. The system consisted of a quaternary pump with a vacuum degasser, an autosampler, a thermostated column compartment and a multiple-wavelength detector. Chromatography was performed at 30 °C using a Zorbax Eclipse XDB-C_8 column (5 μm, 80 Å, 150 mm × 4.6 mm i.d.; Agilent Technologies), equipped with a Wakosil II 5C18-RS guard column (5 μm, 10 mm × 4 mm, SGE, Austin, TX, USA). One hundred microliters of urine, bile and pre-treated samples of plasma and perfusate, respectively, were injected. The mobile phase A was composed of acetonitrile, methanol and 15 mM H_3PO_4 in water pH 2.3 (25:5:70, v/v/v), the mobile phase B was acetonitrile, methanol and 15 mM H_3PO_4 in water pH 2.3 (45:5:50, v/v/v) and the mobile phase C was methanol and water (80:20, v/v). The time program of gradient was: 0–22 min 100% of phase A, 22.01–45 min 100% of phase B and 45.01–55 min 100% of phase C. The flow-rate was 0.8 and 1.1 ml/min in the time period of 0–45 and 45.01–55 min, respectively. Fractions were collected by a fraction collector FC 204 (Gilson, Middelleton, USA) within 1–18 min in the 0.25 min period and in the time period of 18.01–55 min in the 1 min period. The detection was effected by a multiple-wave length detector with UV absorbance at 240 nm and by a liquid scintillation counter Rackbeta 1219 (LKB, Turku, Finland) after taking the fractions and by adding a scintillation cocktail to them.

2.5. Standard solutions

The stock solution of Q with a concentration of 0.05 mg/ml was prepared by dissolving 10 mg of Q in methanol.

Drug-spiked plasma standards were prepared by adding the appropriate volumes of the stock solution to 1.0 ml of plasma (drug-free) to produce a concentration range 0.65–5.00 μg/ml.

3. Results

Fig. 2 shows a comparison of the chromatogram of the non-radiolabelled Q with UV absorbance monitored at 240 nm (a) and the radiochromatogram of the radiolabelled compound (b) in methanol solution. HPLC profile of the non-radiolabelled Q (UV detection) was practically identical with that of ^{3}H-Q (radiometric detection) both in the methanol solution and biological samples, respectively.

^{3}H-Q was pre-purified by HPLC immediately before the biological experiments. The purity was checked on the HPLC system under the same conditions as described above. The radiochromatogram (Fig. 2b) shows that the traces of impurities appear at the retention time period of 17 and 27 min (both together <1% of total activity).

Table 1 shows the recovery of Q extraction in rat plasma. Spiked samples of plasma were prepared by adding known amounts of Q to blank plasma and were extracted as described above. The values represent the ratio of radioactivity eluted by organic solvents from SPE cartridge (directly measured after elution) to total radioactivity (sum of radioactivity in the elution stage and radioactivity measured in a non-retained fraction in the washing step—aqueous phase). No ra-

Table 1
Recovery of Q extraction in rat plasma (mean determined concentration (%) with S.D.) of mean

Added Q concentration (μg/ml)	Recovery ($n = 5$) mean ± S.D. (%)
0.65	99.57 ± 0.04
0.90	99.66 ± 0.02
4.00	99.81 ± 0.02

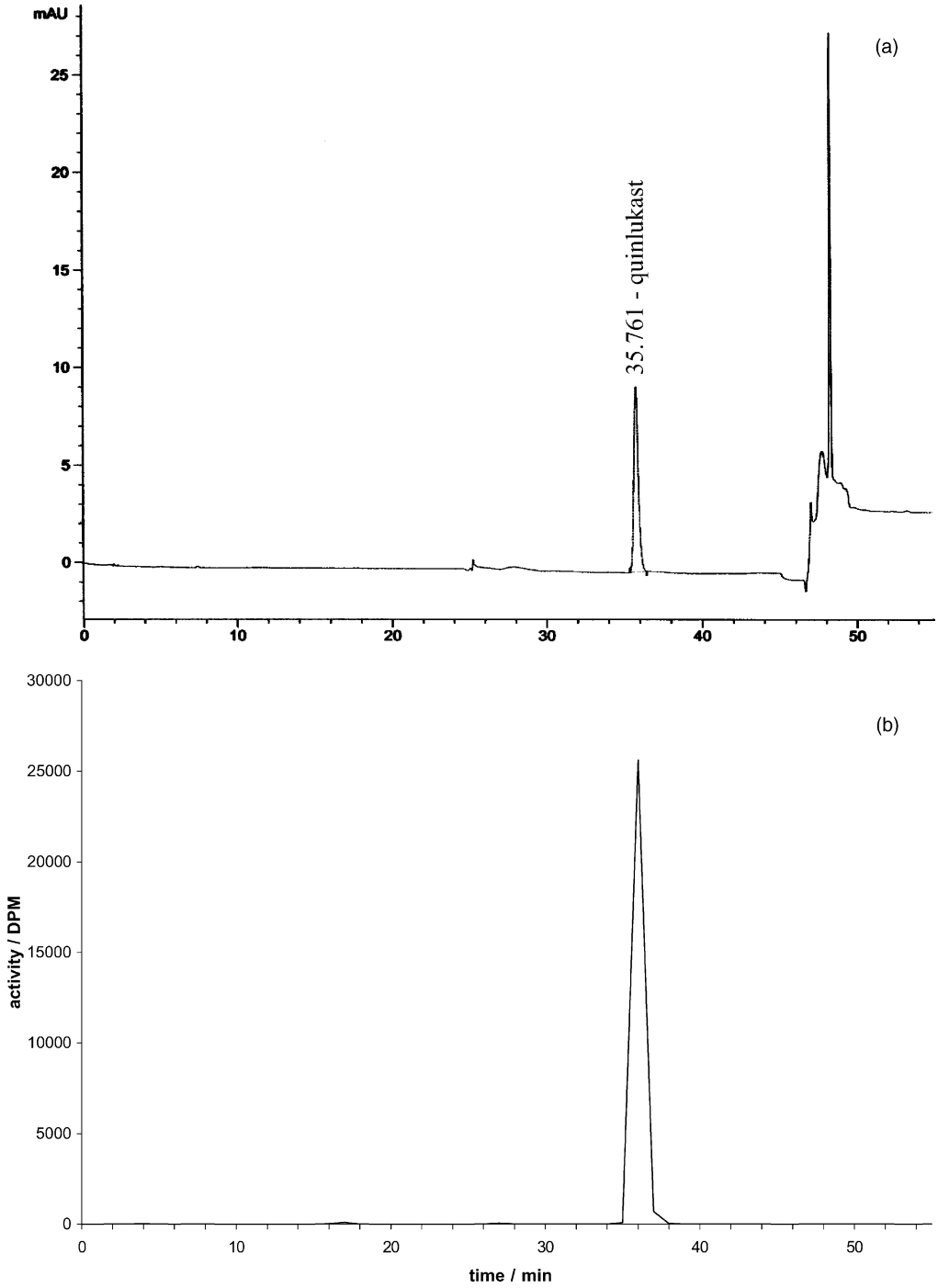

Fig. 2. Example of chromatogram of non-radiolabelled Q with UV absorbance at 240 nm (a) and radiochromatogram of methanol solution of radiolabelled drug (b) using a Zorbax Eclipse XDB-C_8 column (5 μm, 80 Å, 150 mm × 4.6 mm i.d.; Agilent Technologies). The comprehensive conditions of the analysis are described in Section 2.

Table 2
Assay precision and accuracy of the determination of Q in rat plasma (mean determined concentration (μg/ml) with S.D. of mean and relative standard deviation (R.S.D.))

Concentration added (μg/ml)	Within-day ($n = 6$)		Day-to-day ($n = 12$)	
	Concentration found (mean ± S.D.) (μg/ml)	R.S.D. (%)	Concentration found (mean ± S.D.) (μg/ml)	R.S.D. (%)
1.00	1.03 ± 0.01	1.0	0.99 ± 0.02	2.5
2.00	1.93 ± 0.01	0.6	1.88 ± 0.02	1.3
5.00	5.11 ± 0.06	1.2	5.02 ± 0.06	1.1

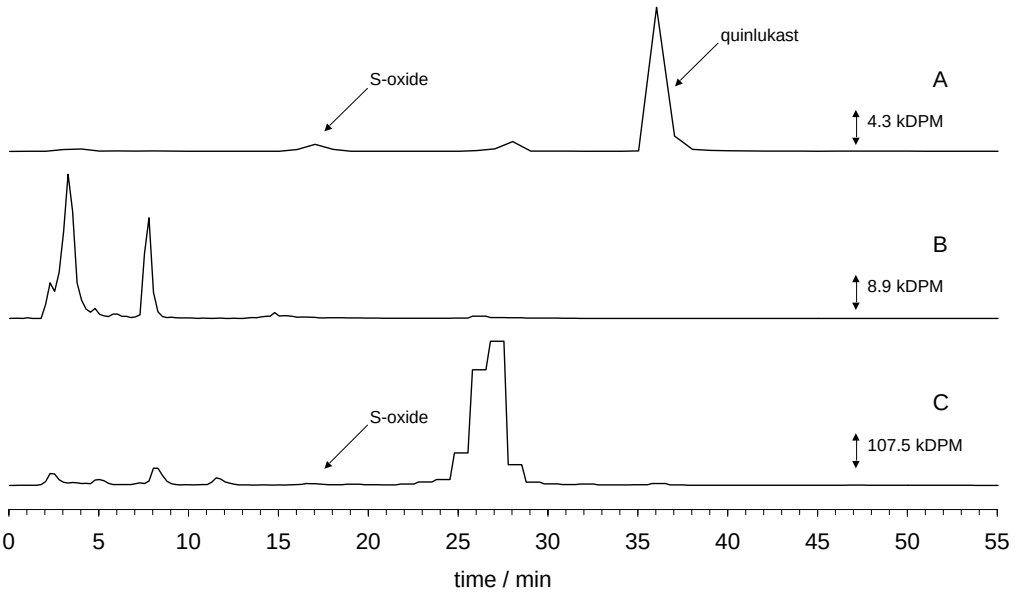

Fig. 3. Examples of radiochromatograms of plasma 120 min after i.v. administration to rats (A), urine withdrawn at the end of the experiment (B) and bile collected in time interval 0–30 min after i.v. administration to rats (C), using a Zorbax Eclipse XDB-C_8 column (5 μm, 80 Å, 150 mm × 4.6 mm i.d.; Agilent Technologies). The comprehensive conditions of the analysis are described in Section 2. All the data correspond to 1 ml of biological fluids.

dioactivity was detected onto the cartridge after the elution.

The precision of this method was determined by assaying a sufficient of aliquots of a homogenous sample so that statistically valid estimates of a standard deviation or relative standard deviation could be calculated [14]. Within- and between-day precisions are presented in Table 2.

The examples of HPLC analyses of plasma, urine and bile are shown in Fig. 3. All the data correspond to 1 ml of biological fluids so that the comparison of the radioactivity profiles in the analyzed biological fluids would be possible.

4. Discussion

Even in the early stage of drug development, the pharmacokinetic and pharmacodynamic consequences of drug metabolism should be included in the general approach. In metabolic studies, HPLC technique is preferred over other methods as it offers specific advantages, such as a possibility of direct analysis of complex mixtures, reproducibility, flexibility, etc. However, such technique requires knowledge of drug metabolic pathways and isolation of individual metabolites prior to the analysis. However, such information is usually lacking in the course of a new

drug development. On the other hand, an employment of radiolabelled drugs enables a very sensitive and accurate counting of radioactivity and, in addition, other non-radiolabelled endogenous and exogenous substances do not interfere in the determination. To be useful, the radiolabelled drug must act in the same way as the non-radiolabelled species in the system of interest. For this reason, Q isotopically labelled with ^{3}H (when one hydrogen atom in the drug structure is replaced by its radioactive isotope tritium) has been used in the present study.

A solid phase extraction procedure was developed for Q in plasma and perfusion medium determination. Discovery DCS-18LT, DSC-8 and DSC-PH columns were tested. In order to increase the efficacy of the extraction procedure to the maximum, Discovery DCS-18LT were selected as the most suitable columns. The recovery of Q extraction in rat plasma achieved on DSC-18LT is presented in Table 1. The results showed that practically all radioactivity was eluted from the cartridge.

At the HPLC analysis of a biological sample which contains a strongly lipophilic parent drug and much more hydrophilic metabolites, an analytical problem arises provided that an isocratic elution is used. In preliminary experiments, a mobile phase with large elution strength was used at the start of analysis; thus polar metabolites present in bile and urine were not separated sufficiently. For that reason the polar mobile phase A was used for the effective separation of polar metabolites and for the elution of S-oxide. The mobile phase was changed to B after 22 min and Q was eluted separately. From 45th min the mobile phase C was used for the elution of compounds with a higher degree of lipophilicity, e.g., dimmers. Under the chromatographic conditions described in Section 2, Q and its S-oxide gave peaks with t_R 35.7 and 16.5 min, respectively. No peak of other metabolites interfered with either peak.

All the determinations were based on radiometric detection. UV detection was used only for comparison of synthetic standard retention times with radiometrically determined t_R of both Q and its S-oxide, present in biological fluids. Quantification of Q and its metabolites was performed on the basis of the known specific activity with regard to the linear dependence of concentration of radiolabelled compound on measured radioactivity. Concentrations of Q and its metabolites were calculated from measured radioactivity. If the radiolabel is still present in the metabolite structure, molar activity of the parent drug and that of the metabolite was the same. The exception is a hypothetical cleavage of a drug molecule, where some non-radiolabelled fragments may arise and thus only the radiolabelled part of the drug structure is determined.

The limit of the detection was calculated to be three times equal the standard deviation of noise ratio, based on the data obtained during the analysis of Q (1.0 μg/ml). The value of this limit was 0.005 μg/ml of Q. The limit of quantification was 0.017 μg/ml of Q. Statistical fluctuation of radioactivity background represented a detection limit for ^{3}H-Q determination. To increase the sensitivity of the analysis, a higher specific activity of Q should be used.

Both within-day and day-to-day precisions were examined (Table 2). The within-day precision was calculated on the basis of the analyses of six rat plasma samples of three Q concentrations. The day-to-day reproducibility was tested during a four-week period. The measured concentrations with standard deviations of mean (S.D.) and relative standard deviations (R.S.D.) are presented in Table 2; all R.S.D. values were less than 5%.

Contrary to in vitro experiments [7,8], only traces of S-oxide of Q were detected in plasma and bile. The reason of this fact is probably a rapid conjugation of this compound in in vivo conditions. Structures of other metabolites, showed in plasma, urine and bile, were not investigated in our project. In the metabolism of a structurally similar drug—montelukast—six metabolites and their diastereomers (including S-oxide) were determined as products of oxidation, conjugation and hydroxylation [15]. We presume that Q could be metabolized by similar metabolic pathways.

5. Conclusion

A combination of a radioactive detection with HPLC separation represents a powerful tool in both pharmacokinetic analysis of new drugs and characterization of their unknown metabolites. Application of this method enables to obtain a more detailed picture of the Q metabolism in rats. The described method is available for the determination of Q and its metabolites in a biological material. After withdraw-

ing the bile and urine samples, their pre-treatment is not necessary; these samples can be directly analyzed using HPLC with radiometric detection. Samples of plasma and perfusate can be prepared by solid phase extraction.

Acknowledgements

The Research Centre Project LN00B125 of the Czech Ministry of Education, Youth and Sports supported this investigation. We wish to thank VUFB (Leciva, Prague, the Czech Republic), especially Dr. Kuchar, for generous gifts of the reference substances of Q and its S-oxide.

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