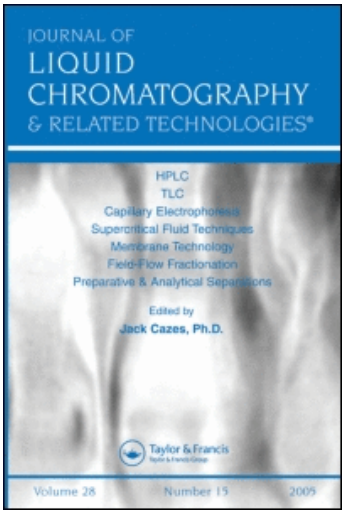

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF KEBUZONE AND ITS METABOLITES IN THE SAMPLES OF ERYTHROCYTES, PLASMA, AND WHOLE BLOOD

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ABSTRACT

Within the framework of behaviour study of non-steroidal anti-inflammatory drugs of different structural types kebuzone was analysed as a representative of the 3,5-pyrazolidinedione group. High-performance liquid chromatographic method has been developed to determine kebuzone and its three metabolites in erythrocytes, plasma and whole blood samples. Isolation of kebuzone from these samples has been optimized with respect to transformation of kebuzone. Samples of erythrocytes, plasma and whole blood were deproteinated, but before this adjustment it was necessary to carry out haemolysis of erythrocytes. The supernatant was chromatographed on a glass column of SGX C-18 (150mm x 3.3mm I.D.) using methanol and water with final apparent pH 2.7 as the mobile phase pumped at a flow-rate of 0.5 ml/min and ultraviolet detection at 247 nm. The method employs an internal standard resulting in good accuracy and precision. The method has been successfully applied to a 2-h pharmacokinetic study of kebuzone in rabbits.

INTRODUCTION

Kebuzone [4-(3-oxobutyl)-1,2-diphenyl-3,5-pyrazolidinedione] is a non-steroidal anti-inflammatory drug successfully used in the treatment of rheumatic diseases. Kebuzone has also a prominent antiuratic effect [1,2]. Several methods have been developed for the determination of kebuzone [1-16]. Various analytical techniques have been used for the assay, such as spectrophotometry [3-6], coulometric method [7], paper chromatography(PC) [1], thin-layer chromatography(TLC) [2,8,9], gas chromatography(GC) [10,11] and high-performance liquid chromatography(HPLC) [12-16]. HPLC determination of 88 drug (including kebuzone) with potential toxicity was described by Eigendorf [12-14]. Dependence of retention characteristics of kebuzone and other 3,5-pyrazolidinediones on eluent pH in reversed-phase HPLC has been published [15, 16]. Determination of kebuzone in biological material has been performed using GC[10, 11], PC [1] and TLC [2,8,9]. PC and TLC methods [1,2,8,9] were used to observe kebuzone and its metabolites (Fig. 1). In paper [2] analytical characteristics of metabolites after its separation by TLC, isolation, clean-up and analysis of spectral methods were compared with the characteristics of synthetically prepared metabolites.

The aim of the present study was to develop a procedure to isolate kebuzone and its metabolites from three different biological matrices - whole blood, erythrocytes and plasma. Optimizations of these biological sample preparations and the analytical procedures were intended to assay kebuzone in the above-mentioned biological matrices using unified HPLC conditions. This method has been applied to the determination of kebuzone in samples arising from a pharmacokinetic study investigating drug transport in the organism of some representative non-steroidal anti-inflammatory drugs (kebuzone represents the 3,5-pyrazolidinedione group).

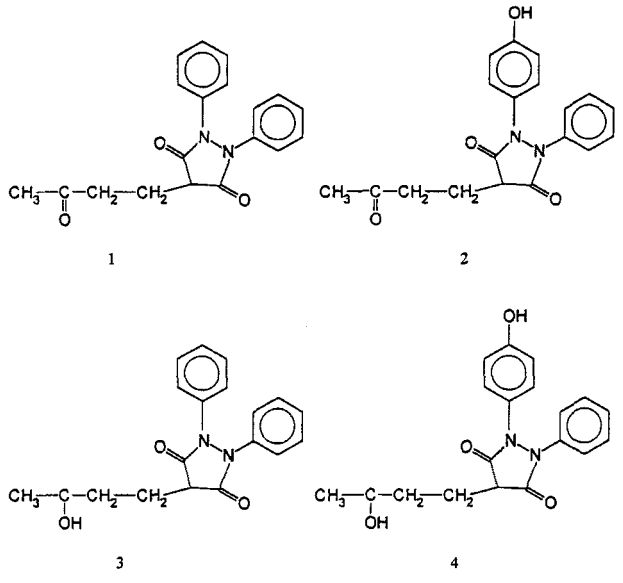

Fig. 1 Structual formulae of kebuzone and its metabolites. 1 = kebuzone, 2 = *p*-hydroxykebuzone, 3 = γ-hydroxykebuzone, 4 = *p*-hydroxy-γ-hydroxykebuzone

MATERIALS AND METHODS

Reagents and chemicals

Kebuzone, Ketazon Spofa inj. and methaqualone (internal standard, IS) were supplied by Léčiva (Prague, Czech Republic). The metabolites *p*-hydroxykebuzone(p), γ-hydroxykebuzone(γ) and *p*-hydroxy-γ-hydroxykebuzone(di) were gifts from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Propyl gallate and ascorbic acid were obtained from Lachema (Brno, Czech Republic) and Farmakon (Olomouc, Czech Republic), respectively. Methanol, acetone, methylene chloride and hydrochloric acid (35%) were also

obtained from Lachema (Brno, Czech Republic) and perchloric acid was from Merck (Darmstadt, Germany).

Kebuzone and IS were PhBs 4 grade, methanol was HPLC grade, and all other chemicals were analytical-reagent grade. Water was doubly distilled.

Chromatographic system

The HPLC system consisted of a Model 8500 Varian pump, Varichrom UV-VIS detector (both: Varian, Palo Alto, CA, USA) and a SP 4100 integrator (Spectra Physics, Santa Clara, CA, USA). Analytical samples were introduced onto the column using a model LCI 30 injection valve (Laboratory Instruments, Prague, Czech Republic) with a 20 µl (or 10 µl) loop. The analytical glass column contained Separon SGX C-18 (150x3.3 mm I.D., 5 µm)(Tessek, Prague, Czech Republic). The mobile phase was a mixture of methanol - water (50:50, v/v) with a final apparent pH of 2.7 adjusted with 5% perchloric acid solution. The flow rate was set at 0.5 ml/min. The UV absorbance of the column effluent was monitored at 247 nm.

Standard solution

Working standards for kebuzone were prepared by dilutions of 25 mg/ml (in methanol) of the stock standard to aliquot concentrations.The stock solutions of kebuzone metabolites (2 mg/ml) and the internal standard (1mg/ml) were prepared in methanol.

Biological sample

Sample of whole blood, erythrocytes and plasma were obtained from the Department of Pathological Physiology, Faculty of Medicine, Charles University

(Hradec Králové, Czech Republic). Here also three rabbits were treated intravenously with the Ketazon Spofa inj. preparation in a 50 mg/kg dose, and the blood samples were withdrawn in the pharmacokinetics study. Samples were withdrawn 3, 6, 15, 30, 60 and 120 min after drug administration.

The withdrawn heparinized rabbit blood was placed in a 10-ml test tube. A portion of each sample of the withdrawn blood was left for analysis (as the sample of whole blood), and for the other portion of the blood, the erythrocytes were isolated from the plasma by centrifugation at 1500 *g* for 10 min. The samples of whole blood, erythrocytes and plasma were immediately frozen.

Sample preparation

Erythrocytes and whole blood. A 0.5 ml volume of sample was pipetted into a 10-ml glass-stoppered centrifuge test tube, 5 µl of the internal standard solution was added and the sample was haemolyzed by adding 0.9 ml of water. The sample was shaken for 5 min, placed in an ultrasonic bath for 5 min, and left at room temperature for 5 min. To the sample 2 ml of acetone was added and the sample was shaken for 5 min, centrifuged at 1930 *g* for 5 min, the supernatant was separated and thickened to a volume of 0.5 ml under a gentle stream of nitrogen. A 20 µl aliquot of the thickened supernatant was injected onto the HPLC column.

Plasma. A 0.5 ml volume of plasma sample was pipetted into a 10-ml glass-stoppered centrifuge test tube, 5 µl of the internal standard solution and, after shaking, 0.75 ml of acetone were added. Then the procedure continued in the same manner as described for the whole blood and erythrocytes samples.

Preparation of the standard curves

Calibration standards were prepared by adding 10 µl of the appropriate working standard and 5 µl of the internal standard to 0.5 ml of whole blood, 0.5 ml of

erythrocytes and 0.5 ml of plasma. Five calibration concentrations of kebuzone were used for the standard curve in each matrix - 2, 10, 20, 30 and 40 μg/ml of isolated erythrocytes, 100, 200, 300, 400 and 500 μg/ml of plasma and 50, 100, 150, 200 and 300 μg/ml of whole blood. Sample preparation was carried out as described above.

Concentrations of kebuzone were calculated from the linear regression equation of the calibration curve constructed by plotting the peak area ratio (y) of kebuzone and the internal standard versus the concentration of kebuzone (x).

RESULTS AND DISCUSSION

In the elaboration of HPLC method for the analysis of kebuzone there was a need to allow a "relatively easy" transformation of kebuzone. Kebuzone in aqueous and methanolic solution[17,18] gives in to very easy and rapid transformation. Series of the factors (e.g. light, warmth, change in pH, etc.) [19,20] produce transformation owing to oxidation and hydrolytic processes. There occur [9,18,21] either transformation on the pyrazolidinedione ring (e.g. it can be oxidized to 4-hydroxyderivate of kebuzone) and/or the decomposition of the pyrazolidinedione ring (hydrazobenzen, azobenzen are formed). In the present paper undesirable transformation was observed, which is analogous to the difficulties described in papers [9,22,23].

Isolation technique

The original aim of our work was to develop a liquid-liquid extraction procedure to isolate kebuzone from three different biological matrices - whole

blood, isolated erythrocytes and plasma. The samples were adjusted as follows: To 0.5 ml of whole blood (or erythrocytes) in a glass-stoppered centrifuge test tube, 50 μg standard solution of kebuzone and 5 μl of the internal standard solution were added and the sample was haemolysed . After haemolysis the sample was acidified with hydrochloric acid (5 mol/l), and after shaking kebuzone was extracted to 6 ml methylene chloride for 5 min. Methylene chloride was separated and evaporated to dryness under a gentle stream of nitrogen. The dry residue was reconstituted with methanol and 10 μl was injected to the HPLC column. The same procedure, but without haemolysis, was used when isolating kebuzone from the plasma sample. Fig. 2 shows that in chromatogram A there is an undesirable peak Y (t_R 5.6 min) in addition to the peak of kebuzone(K) and peaks of blood residues. Peak Y was recorded on the chromatogram in a modification of this extraction process (alteration of acid and its strength, use of another extraction solvent, *e.g.* chloroform, etc).

To stabilize kebuzone, an antioxidant was added to the samples of the biological matrix. Of the antioxidants, ascorbic acid and propyl gallate were effective.

Ascorbic acid (100 μl 1% methanol solution added to 0.5 ml volume of each biological sample) prevented transformation of kebuzone during the extraction step in all three biological samples. But after solution of dry residues of the samples in methanol, slow transformation of kebuzone gradually. Occurred on the HPLC chromatogram (Fig 2., chromatogram B) a small peak Y developed 15 min after solution of the residues, which gradually advanced on repeated injection.

Only propyl gallate (PG) (100 μl 0.5% methanol solution added to a 0.5 ml volume of each biological sample) stopped the transformation of kebuzone. After solution of dry residues samples in methanol the kebuzone peak was standing still after 24 hours. A disadvantage of the use of propyl gallate as the stabilizing agent

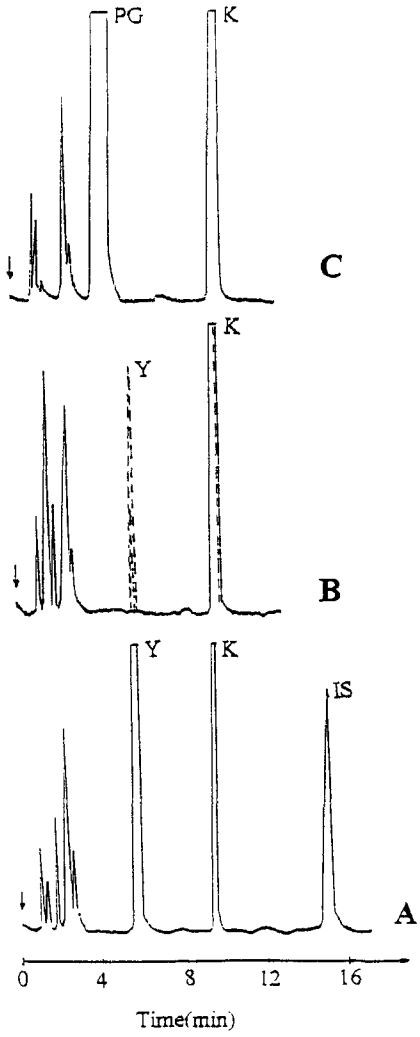

Fig.2 Effect of stabilizing agents on kebuzone(K) during its extraction into methylene chloride. A - chromatogram without the addition of the stabilizing agent (Y - undesirable peak), B - chromatogram with the addition of ascorbic acid solution (Y - undesirable peak), C - chromatogram with the addition of propyl gallate (PG) solution

was its very good recovery (85.3%) into methylene chloride. See the bulky peak (PG) in chromatogram C in Fig 2. An added smaller amount of propyl gallate to the sample was not effective on examination.

This extraction process (with an addition of propyl gallate solution to the sample before its extraction) is useful for the isolation and examination of HPLC analysis of kebuzone from the samples of the whole blood, isolated erythrocytes and plasma. It is true that then the peak of propyl gallate can interfere with the peaks of kebuzone metabolites. There is no doubt that it is disadvantageous in the pharmacokinetic study of kebuzone. Therefore an attempt was made to use deproteination of samples instead of extraction into an organic solvent.

Methanol, perchloric acid (30%), ammonium sulfate, acetonitrile and acetone were tested as protein precipitants. Acetone proved to be the best of the protein precipitants tested for the determination of kebuzone in the whole blood sample, isolated erythrocytes sample and plasma sample because the undesirable peak Y didnot appear in the chromatogram even after 24 hours. When using acetone to deproteinate the biological matrix, samples must be evaporated under a gentle stream of nitrogen until all acetone is removed from the separated supernatant, because acetone absorbs ultraviolet light up to 330 nm.

In order to increase the efficacy of the isolation procedure, it was necessary to carry out the haėmolysis of erythrocytes in the samples of whole blood and isolated erythrocytes. The recovery values are listed in Table 1.

Chromatography

Under chromatographic conditions described in the Experimental part, kebuzone, *p*-hydroxy-*γ*-hydroxykebuzone, *p*-hydroxykebuzone, *γ*-hydroxykebuzone and methaqualone (I.S.) were completely separated with retention times of 9.2, 3.9,

TABLE 1

RECOVERY OF KEBUZONE IN RABBIT ERYTHROCYTES, PLASMA AND WHOLE BLOOD

Biological material	Added conc. (μg/ml)	Recovery (n=5) mean ± S.D. (%)
Erythrocytes	2.0	67.7 ± 2.9
	10.0	68.8 ± 2.7
	40.0	70.0 ± 3.1
Plasma	150.0	93.1 ± 2.1
	300.0	94.9 ± 2.2
	450.0	95.0 ± 2.1
Whole blood	50.0	79.0 ± 2.4
	150.0	79.5 ± 2.2
	250.0	80.2 ± 2.3

4.4, 8.8 and 15.0 min, respectively. No interfering peaks of the retention times of analysed compouds were seen in blank samples of whole blood, isolated erythrocytes a plasma. Fig. 3 and 4 show the representative chromatograms of blank (A), control (B) and dosed (C) rabbit whole blood and erythrocyte samples, respectively.

Quantitative determination of kebuzone was performed. Metabolites of kebuzone were not quantified, because in a 2-hour pharmacokinetic study they were not detected. The calibration curves of kebuzone displayed good linearity

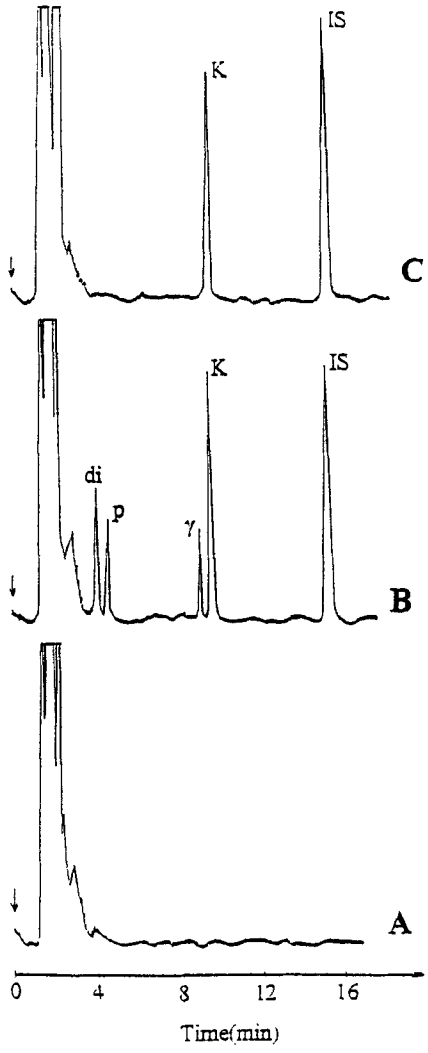

Fig.3 Chromatograms for kebuzone(K), its metabolites / *p*-hydroxy-*γ*-hydroxy-kebuzone(di), *p*-hydroxykebuzone(p) and *γ*-hydroxykebuzone(γ) / and the internal standard (IS) in rabbit erythrocytes. A-blank samples, B-control samples spiked with standard solution of kebuzone (10 μg/ml) and its metabolites (and IS), C-30-min sample from a rabbit given a single dose of 50 mg/kg kebuzone.

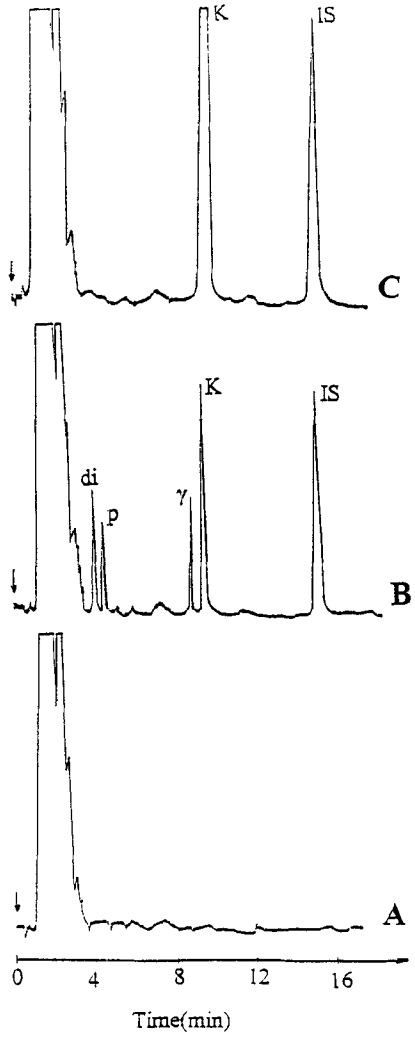

Fig.4 Chromatograms for kebuzone(K), its metabolites / *p*-hydroxy-*γ*-hydroxykebuzone(di), *p*-hydroxykebuzone(p) and *γ*-hydroxykebuzone(γ) / and the internal standard (IS) in rabbit whole blood. A-blank samples, B-control samples spiked with standard solution of kebuzone (10 μg/ml) and its metabolites (and IS), C-30-min sample from a rabbit given a single dose of 50 mg/kg kebuzone.

TABLE 2

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS OF KEBUZONE IN ERYTHROCYTES, PLASMA AND WHOLE BLOOD

Biological material	Regression equation $y = ax + b$	Correlation coefficient r
Erythrocytes	$y = 0.0794x + 0.0060$	0.9998
Plasma	$y = 0.0794x + 1.0000$	0.9985
Whole blood	$y = 0.0682x + 0.1373$	0.9992

over the range examined in each biological matrix. Their regression equation and correlation coefficients are given in Table 2.

Both within-day and day-to-day precision and accuracy of standard curves are examined. Within-day precision was calculated from the analysis of six samples in each matrix of four concentrations of kebuzone. Day-to-day reproducibility was investigated during a four-week period. Measured concentrations and coefficients of variation (C.V.) are presented in Table 3; the C.V. values were all less than 5%.

The detection limit for kebuzone was 3 μg/ml in whole blood and plasma and 10 μg/ml in erythrocytes. The limit of quantification was 10 μg/ml in whole blood and plasma and 22 μg/ml in erythrocytes. The sensitivity of this HPLC assay of kebuzone depends on the adjustment of the sample of protein precipitation. In papers [12-16] in HPLC analysis of kebuzone only some retention characteristics (retention times, capacity factors) are stated, but none of these paper introduces the validation of the kebuzone HPLC assay (or its HPLC determination in biological samples) as the present paper.

TABLE 3

ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF KEBUZONE IN ERYTHROCYTES, PLASMA AND WHOLE BLOOD

Conc. added (μg/ml)	Within-day (n=6)		Day-to-day (n=12)	
	Concentration found (mean ± S.D)(μg/ml)	C.V. (%)	Concentration found (mean ± S.D)(μg/ml)	C.V. (%)
Erythrocytes				
4.0	4.2 ± 0.1	3.3	4.3 ± 0.2	4.2
16.0	15.8 ± 0.4	2.4	16.4 ± 0.5	3.0
24.0	24.6 ± 0.4	1.2	24.8 ± 0.6	2.3
36.0	36.5 ± 0.7	1.8	37.0 ± 0.8	2.2
Plasma				
150.0	154.5 ± 2.2	1.4	153.1 ± 2.1	1.4
250.0	252.0 ± 2.6	1.0	251.5 ± 2.9	1.1
350.0	354.2 ± 3.2	0.9	355.0 ± 3.7	1.1
450.0	447.3 ± 4.1	0.9	449.1 ± 5.2	1.1
Whole blood				
60.0	61.8 ± 1.1	1.8	62.4 ± 1.4	2.2
120.0	122.3 ± 2.0	1.6	123.2 ± 2.5	2.1
180.0	183.4 ± 2.7	1.5	181.9 ± 2.8	1.6
240.0	244.0 ± 4.1	1.7	244.8 ± 4.0	1.6

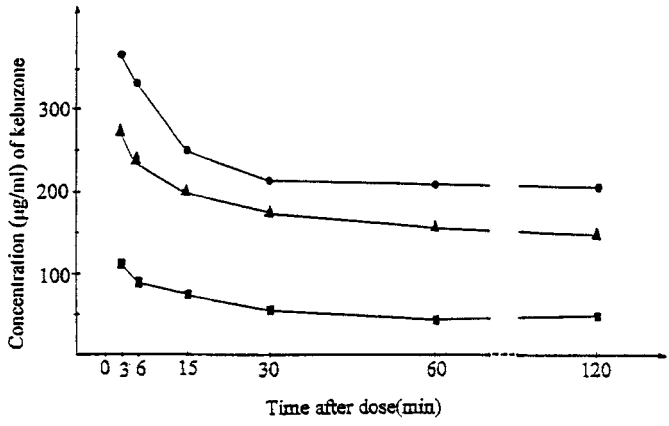

Fig.5 Concentration of kebuzone in erythrocytes (■), plasma (●) and whole blood (▲) of rabbit following intravenous administration of Ketazon inj.

Pharmacokinetic study

In order to investigate drug levels in the transport of some representative non-steroidal anti-inflammatory drugs in the organism, representatives of different structural types were selected. Kebuzone, a representative of 3,5-pyrazolidine-diones, was determined similarly as ibuprofen [24] and acetylsalicylic acid[25], representatives of profens and salicylates, respectively.

In a 2-hour pharmacokinetic study the samples of whole blood, isolated erythrocytes and plasma were analysed and the amounts of kebuzone determined. The observed blood, erythrocytes and plasma levels of kebuzone (Fig.5) with a simultaneous examination of the distribution of kebuzone between erythrocytes and plasma for three laboratory rabbits are presented. Fig.6 compares the concentration obtained by the sum of the concentrations found in erythrocytes and plasma (related to 1 ml of withdrawn blood with regard to the haematocrit value) and the

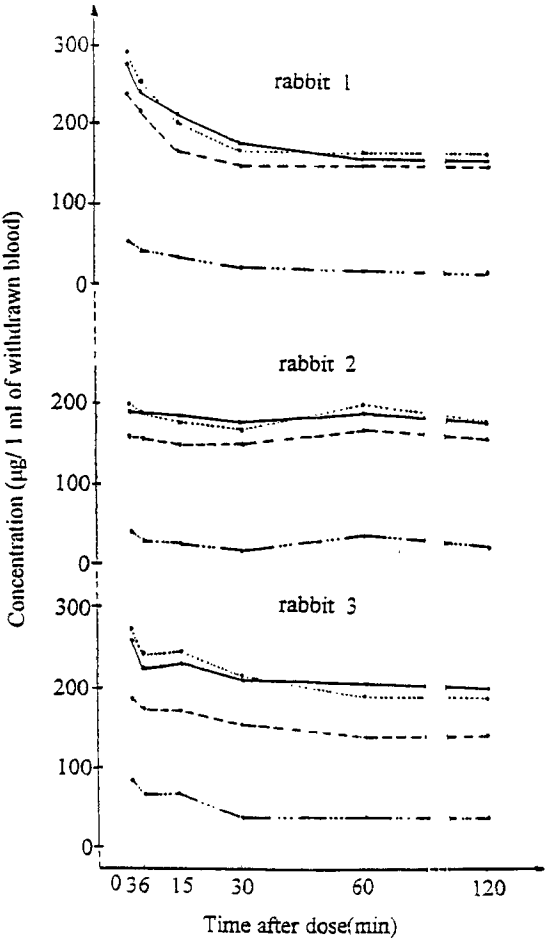

Fig.6 Distribution of kebuzone between erythrocytes and plasma in 1 ml of withdrawn blood during 2h after intravenous administration. (— ··· —) erythrocytes; (- - - - -) plasma; (. . . . . .) sum of the levels; (———) level found in the analysis of the whole blood sample.

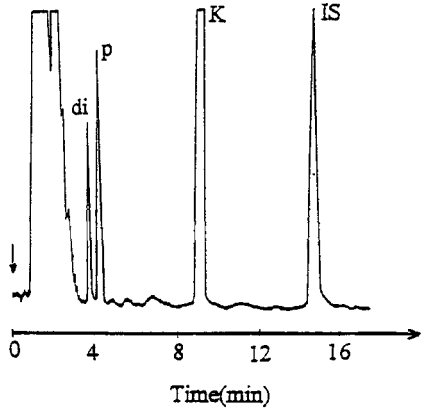

Fig.7 Chromatogram for kebuzone(K), its metabolites / *p*-hydroxy-*γ*-hydroxy-kebuzone(di) and *p*-hydroxykebuzone(p) / and the IS in rabbit whole blood 24-h sample from a rabbit given a single dose of 50 mg/kg.

concentration found in whole blood. The comparison of kebuzone levels in plasma, erythrocytes and whole blood shows that directly after administration kebuzone is already evenly distributed between erythrocytes and plasma and its levels almost do not change in both matrices. 20% of released kebuzone is demonstrated in erythrocytes and 80% in plasma . The metabolites of kebuzone were demonstrated in no biological matrix in the course of a 2-hour pharmacokinetic study. Althrough for the purposes of our cooperation with the Faculty of Medicine the determination of the levels of kebuzone during two hours in this biological matrix was sufficient, the samples of blood were still withdrawn 10 and 24 hour after drug administration. It was found that *p*-hydroxy-*γ*-hydro-xykebuzone and *p*-hydroxykebuzone were detected in samples of whole blood (Fig. 7) and plasma, but not in erythrocytes samples. *γ*-hydroxykebuzone was not detected in the samples of any biological matrix withdrawn after 24 hour.

Conclusion

In the present paper, a method using HPLC for the analysis of kebuzone and its metabolites in the samples of erythrocytes, plasma and whole blood was developed. HPLC analysis of kebuzone and its metabolites has not been described in the literature yet. If we want to determine only kebuzone (without its metabolites), it can be used for the adjustment of blood, erythrocytes and plasma sample extraction with methylene chloride after adding propyl gallate solution (as the stabilizing agent). In the simultaneous determination of kebuzone and its metabolites it was suitable to perform, before HPLC analysis, precipitation of proteins in the mentioned samples and inject the supernatant to the column. This HPLC method makes it possible to obtain a more detailed picture of the distribution of kebuzone in blood between erythrocytes and plasma and to contribute to a more objective view of the results of the analysis as dependent on the isolated biological matrix. It can be generally stated that whole blood provided more objective information about drug levels than plasma, though it is a more complicated biological matrix. Besides determining kebuzone levels in blood and distribution of kebuzone between erythrocytes and plasma, other physiological or pathophysiological parameters were investigated in laboratory rabbits by our collaborator from the Faculty of Medicine.

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REFERENCES

1. M. Queisnerová, O. Siblíková, M. Veverková, K. Macek, O. Němeček, Českoslov. Farm., 16: 219-222 (1967)

2. M. Queisnerová, J. Grimová, Českoslov. Farm., 21: 325-328 (1972)

3. B. Kakáč, M. Peterková, Českoslov. Farm., 26: 274-277 (1977)

4. M. Peterková, B Kakáč, O. Matušková, Českoslov. Farm., 26: 255-258 (1977)

5. Ž. Bezáková, M. Bachratá, M. Blešová, L. Kňažko, Farm. Obzor, 49: 157-167 (1980)

6. M. Pospíšilová, D. Svobodová, Českoslov. Farm., 41: 324-328 (1992)

7 Ch. Herrenknecht, E. Guernet-Nivaud, M. Guernet and C. Guernet, Ann. Pharm. Fr., 43: 557-563 (1985)

8. O. Němeček, K. Macek, M. Queisnerová, Z. J. Vejdělek, Arzneim.-Forsch., 16: 1339-1342 (1966)

9. O. Siblíková, M. Černá, I. M. Hais, Českoslov. Farm., 11:123-126 (1962)

10. A. Scelepcsenyiová, J. Hrivnak, L. Zathurecký, Českoslov. Farm., 25: 400-402 (1976)

11. K. K. Midha, I. J. McGilveray, J. K. Cooper, J. Pharm. Sci., 67: 279-281 (1978)

12. H.-G. Eigendorf, Pharmazie, 43: 287-288 (1988)

13. H.-G. Eigendorf, G. Moschwitzer, R. Budde, Pharmazie, 44: 645-646 (1989)

14. H.-G. Eigendorf, R. Budde, G. Moschwitzer, H. Konig, Pharmazie, 45: 219-220 (1990)

15. Ch. Herrenknecht, D. Ivanovič, E. Guernet-Nivaud, M. Guernet, J. Pharm. Biomed. Anal., 8: 1071-1074 (1990)

16. E. Nivaud-Guernet, M. Guernet, D. Ivanovič, M. Medenica, J. Liq. Chromatogr., 17: 2343-2357 (1994)

17. M.Takács, P. Kertész, E. Weiner, J. Reisch, Arch. Pharm/Weinheim/, 318: 824-832 (1985)

18. R. Wachowiak, Pol. J. Pharmacol. Pharm., 30: 833-843 (1978)

19. M .Liška, M. Zahradníček, Československ. Farm., 18: 380-384 and 438-444 (1969)

20. M .Liška, M. Zahradníček, Československ. Farm., 19: 82-88 (1970)

21. M.Takács, G. Simon, J. Reisch, Arch. Pharm/Weinheim/, 317: 817-823 (1984)

22. K. Macek, J. Chromatogr., 33:332-333 (1968)

23. M.Takács, P. Kertész, J. Reisch, Pharm. Acta Helv., 61: 148-149 (1986)

24. J. Sochor, J. Klimeš, M. Zahradníček, J. Sedláček, J. Chromatogr. B, 654: 282-286 (1994)

25. J. Klimeš, J. Sochor, M. Zahradníček, J. Sedláček, J. Chromatogr., 584: 221-228 (1992)

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