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DETERMINATION OF CIRCULATING ETHYL LOFLAZEPATE METABOLITES IN THE BABOON BY RADIO-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH INJECTION OF CRUDE PLASMA SAMPLES: COMPARISON WITH SOLVENT EXTRACTION AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Circulating ethyl loflazepate metabolites in the baboon were determined, following a single oral administration of the ^{14}C-labelled drug, by radio-high-performance liquid chromatography with injection of crude plasma samples and by selective extraction with thin-layer chromatographic analysis of the radioactive components. Metabolites identified by comparing their chromatographic behaviour with synthetic standards were loflazepate, descarboxyloflazepate and 3-hydroxydescarboxyloflazepate. Loflazepate represented about 70% of the circulating radioactivity; the two other metabolites were present in amounts too small to allow accurate quantification. The parent drug was not present in the blood. Comparison of high-performance liquid chromatography with solvent extraction demonstrated the inaccuracy of the latter to be caused by the conversion of loflazepate to descarboxyloflazepate.

INTRODUCTION

Experiments in vivo and in vitro showed that a major and early step in the biotransformation of ethyl loflazepate (EL, Fig. 1) (Victan), a new anxiolytic drug, was the hydrolysis of the ester bond leading to the formation of the anionic derivative loflazepate (L) [1]. Owing to its chemical instability, L cannot be directly extracted from biological samples, and was generally extracted and quantified after its decarboxylation to descarboxyloflazepate (DCL) [2]. As DCL may also be a metabolite of EL, the conversion of L to DCL should be realized after exhaustive extraction of the latter. As a consequence of

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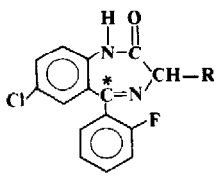
these analytical difficulties, a sensitive and specific high-performance liquid chromatographic (HPLC) method with on-line radioactivity detection and injection of crude biological samples was developed and used for characterization and semi-quantification of plasma metabolites following oral administration of [^{14}C]EL to a baboon. Comparison with data obtained after extraction of plasma samples and thin-layer chromatographic (TLC) analysis of the radioactive components was made.

EXPERIMENTAL

Chemicals

Radioactive [^{14}C]EL, with the position of the radiolabel indicated in Fig. 1, supplied by CEN (Saclay, France), had a specific activity of 40.35 μCi/μmol and a radiochemical purity greater than 98% determined by TLC on silica gel in chloroform—diisopropyl ether—ethanol (70:20:5). Labelled standards used to establish retention times were produced from [^{14}C]EL either by incubation with plasma of rat (L) [1] or by administration to the baboon and preparative extraction of the obtained 0—24 h urine samples at pH 9 with dichloromethane (DCL and 3-OH-DCL) [3]. Unlabelled compounds were provided by A. Hallot (Chemistry Section, Department of Neurobiology, Clin-Midy, Groupe Sanofi, Montpellier, France). All other chemicals, including the solvents, were of at least reagent grade. Except for L, used in 0.05 *M* hydrogen carbonate buffer (pH 9) or crude plasma, all other standards were used in organic solution (acetone or dichloromethane).



R = $COOC_2H_5$ (EL)
R = COO^- (L)
R = H (DCL)
R = OH (3—OH—DCL)

Fig. 1. Structures of ethyl loflazepate and reference compounds. The position of the ^{14}C label is indicated.

Administration of the drug

A female baboon (*Papio papio*) weighing 5 kg was fasted overnight and given a single oral dose (1 mg/kg, 107 μCi/kg) of the drug by gastric intubation. The experimental form was a solution of the drug in acetone—propylene glycol—water (1:4:5). The animal was then placed in a metabolism cage and access to tap water ad libitum. It was housed in air-conditioned accommodation with an ambient temperature of 24—26°C and hygrometry within 40—60%. Fluorescent lighting was provided for a period of 12 h daily and the air was changed twelve to fifteen times per h.

Blood sampling

At prescribed intervals, up to 24 h after dosing, blood samples were withdrawn into vacutainer tubes containing ammonium and potassium oxalate. After removal of aliquots for total blood radioactivity measurement, the collected samples were centrifuged at 5000 *g* for 20 min at 4°C. Plasma was

immediately frozen using liquid nitrogen and stored at −20°C until analysis.

Analytical procedures

Plasma samples were analysed using two techniques. The first involved HPLC with on-line radioactivity detection and direct injection of the crude sample. The second was based on selective extraction and TLC separation of the labelled compounds.

HPLC was performed on a Varian 5000 instrument (Varian, Palo Alto, CA, U.S.A.) equipped with a μBondapak C_{18} (10 μm) stainless-steel column (250 × 4 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and a FLO-ONE/DR on-line radioactivity monitor set for ^{14}C (Radiomatic Instrument and Chemical Co., Tampa, FL, U.S.A.). The mobile phase was distilled water—acetonitrile (50:50) with a flow-rate of 1 ml/min. The radioactivity detector was equipped with a 0.5-ml flow cell and the mobile phase was mixed with 3 ml/min of Lumaflow II (Kontron, Velizy, France) just before entry into the counting cell. Aliquots (10 μl) of crude plasma samples were injected directly into the HPLC system. The retention times of the radioactive peaks were compared with those of authentic standards run under the same analytical conditions. Quantitative data obtained were radioactivity for each peak in dpm, total radioactivity in the sample and relative contribution of each peak to total radioactivity. Plasma samples were de-frozen one by one just before injection and immediately re-frozen after analysis.

Plasma samples (1 ml) were adjusted to pH 9 with 1 *M* sodium hydroxide and extracted six times with 5 ml of dichloromethane. The combined organic extracts were concentrated to 10 ml. The remaining aqueous layers were adjusted to pH 2 and kept for 30 min at 37°C in order to decarboxylate the unstable anionic derivatives. The resulting compounds were extracted at pH 9 with dichloromethane. Aliquots of organic extracts were concentrated to dryness, reconstituted in 0.1 ml of ethanol—dichloromethane (50:50) and spotted on silica gel GF_{254} TLC plates (Merck, Darmstadt, F.R.G.) together with authentic standards. The plates were developed in chloroform—diisopropyl ether—ethanol (70:25:5). Radioactive components on the plate were detected by exposure to Kodak NS 2T X-ray film and non-radioactive reference compounds were detected under ultraviolet light at 254 nm.

Aliquots of blood and plasma samples were counted directly in Biofluor scintillation mixture (New England Nuclear, Boston, MA, U.S.A.). Aliquots of the extracts were evaporated to dryness and reconstituted in the same scintillation mixture. Radioactive TLC spots were scraped off from the plate and counted as a gel in a 5:15 mixture of water and Unisolve 1 (Koch-Light Labs., Colnbrook, U.K.). All radioactivity measurements were made using a Packard 3390 liquid scintillation spectrometer equipped with a Packard 544 Absolute Activity Analyzer.

RESULTS

Plasma and blood concentration versus time curves are presented in Fig. 2. The peak concentration of the circulating radioactivity was observed 3 h after dosing. Each area under the curve (AUC) was determined by the

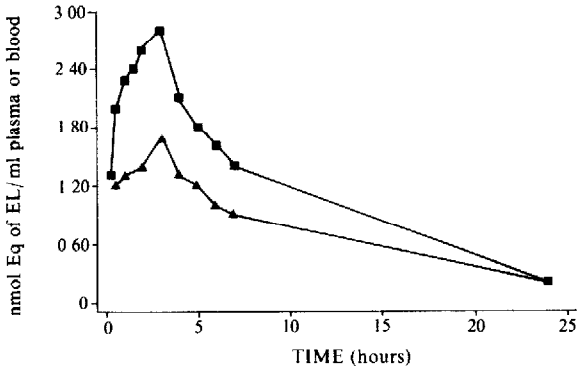


Fig. 2. Concentrations of total circulating metabolites after oral administration of [^{14}C]EL to a baboon. Data expressed as nmol equiv. of EL per ml plasma (■) or blood (▲). AUC blood = 17.7 nmol · h · ml^{-1}; AUC plasma = 27.8 nmol · h · ml^{-1}. The dose of [^{14}C]EL was 4.78 mg (13 μmol).

trapezoidal rule on the individual data using a Tektronix microcomputer. The calculated plasma/blood AUC ratios indicated that a large fraction of the circulating compounds was localized in the plasma.

The results obtained from HPLC runs are presented in Fig. 3. Accurate quantitative data were obtained for L which represented suitable levels of radioactivity for quantitation. Values for DCL and 3-OH-DCL are intended only as a quantitative estimate as their low levels are below the limit of accuracy of the method. Further, as a consequence of column obstruction due to repeated injection of crude plasma samples and the resulting increase in pressure, these two compounds were eluted at the same time during analysis of later plasma samples (Fig. 4). Therefore, the values reported in Fig. 3 represent DCL + 3-OH-DCL concentrations.

From the AUC ratios it was concluded that up to 7 h after dosing L represented about 71% of total plasma metabolites and DCL + 3-OH-DCL about 20%. Trace amounts of unknown radioactive material were also detected in the chromatogram.

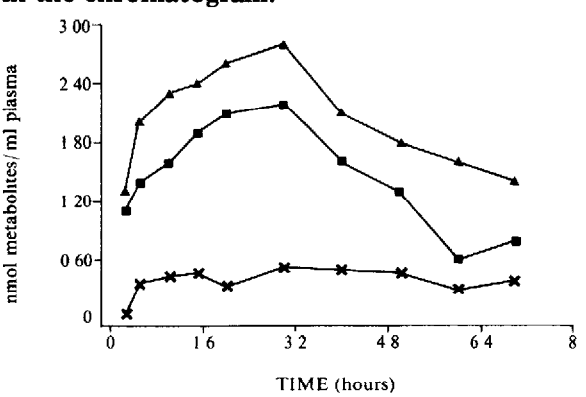


Fig. 3. Plasma concentrations of EL metabolites after oral administration of 4.78 mg (13 μmol) of [^{14}C]EL to a baboon. Data expressed as nmol per ml of plasma. (▲) Total metabolites; (■) L concentrations; (×) DCL + 3-OH-DCL concentrations. AUC (L) = 10 nmol · h · ml^{-1}; AUC (DCL + 3-OH-DCL) = 2.8 nmol · h · ml^{-1}.

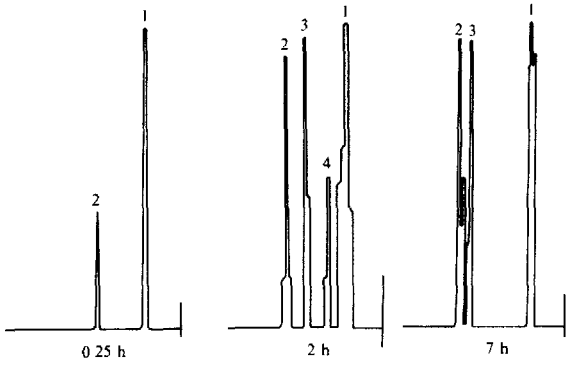


Fig. 4. HPLC separation of plasma metabolites 0.25, 2 and 7 h after oral administration of [^{14}C]EL to a baboon. The figure shows the traces of L (1) with a retention time of 2.50 min, DCL (2) with a retention time of 6, 6.10 and 6.50 min for 0.25, 2 and 7 h plasma sample, respectively, 3-OH-DCL (3) with respective retention times of 5 min (2-h sample) and 6.50 min (7-h samples) and unknown material (4).

TABLE I

PLASMA CONCENTRATIONS (nmol/ml) OF RADIOACTIVE METABOLITES FOLLOWING A SINGLE ORAL ADMINISTRATION OF [^{14}C]EL TO A BABOON

Time (h)	Extraction procedure				HPLC	
	Extractability (%)*	L	DCL	3-OH-DCL	L	DCL + 3-OH-DCL
0.25	92	0.7	0.5	ND**	1.1	0.04
0.5	75	1.1	0.4	ND	1.4	0.36
1	92	1.4	0.6	0.1	1.6	0.44
1.5	67	1.4	0.3	ND	1.9	0.47
2	NA**				2.1	0.33
3	76	1.3	0.6	0.2	2.2	0.53
4	71	0.8	0.5	0.2	1.6	0.50
5	59	0.4	0.4	0.3	1.3	0.47
6	62	0.4	0.3	0.3	0.6	0.29
7	76	0.4	0.3	0.3	0.8	0.40
24	100	0.1	0.05	0.05	NA	NA

*Mean value of the percentage extractability over 7 h = 75%.
**NA = Not analysed; ND = not detected.

Data obtained from the extraction and TLC procedure, together with those obtained from HPLC analysis of crude plasma samples, are reported in Table I. Amounts of L recovered after extraction were, as expected, lower than those recovered from HPLC analysis and, consequently, DCL amounts were higher. The observed differences were explained by the chemical instability of L, which resulted in the formation of DCL whenever biological samples were submitted to freezing and thawing, extraction procedures or other analytical procedures. Hence the levels of the two compounds would be influenced by the uncontrolled degradation of L [4]. Regarding 3-OH-DCL, the amounts extracted and characterized by TLC and those obtained by HPLC analysis were

almost identical. The observed differences can be attributed to the limited accuracy, owing to the low levels of radioactivity investigated.

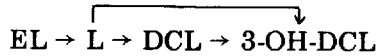
The total radioactivity recovered after dichloromethane extraction was about 75% (mean value) of the radioactivity initially present in the plasma. The radioactivity remaining in the sample can be attributed to unknown material (trace 4 in Fig. 4) or to other polar, non-extractable components.

DISCUSSION

Earlier studies demonstrated the exhaustive biotransformation of EL to L during the crossing of the intestinal wall, in the blood and the liver (first pass effect) [1]. However, owing to its chemical instability, L was indirectly characterized and quantified after its conversion to DCL. As DCL should be a metabolite of EL, pharmacokinetic data were obtained by measuring the total levels of DCL + L after conversion of the latter. In this study, direct evidence of the presence of L in plasma samples was obtained using HPLC with on-line radioactivity detection and direct injection of crude biological samples. The assay revealed that this compound was the major metabolite at all times of the investigation.

In vitro data indicated that 3-OH-DCL was not obtained from incubation at 37°C of EL, L and DCL in crude plasma. Hence the occurrence of the 3-OH-DCL peak in the chromatogram cannot be the result of degradation during handling of the sample. As regards the DCL peak, the following comments can be made. From in vitro studies it was demonstrated that the conversion of L to DCL in plasma following incubation at 37°C was enzymic. Hence a valid judgement of the actual presence of this compound in vivo or on its ex vivo production cannot be made. However, as in this study all blood samples were handled during the same time and in cold conditions (centrifugation at 4°C immediately after the sampling and immediate dipping of plasma samples in liquid nitrogen) and as DCL concentrations increased with time, although slightly, it can be concluded that its formation occurred in vivo.

From this study and previous work [1, 4], the following metabolic affiliation may be proposed:



This would mean that a direct relationship exists between levels of L and of DCL and 3-OH-DCL. Under these conditions L may be regarded as a constant and steady source of DCL and 3-OH-DCL. This behaviour should explain the pharmacokinetic profile of DCL and 3-OH-DCL in plasma, which looks very similar to a conventional perfusion profile, in particular with the absence of a clear peak concentration. Furthermore, limits of detection of the assay procedures were sufficiently low to ascertain the absence of the parent drug in blood.

Comparison of data obtained from direct HPLC analysis and from a classical extraction procedure demonstrated the limited accuracy of the latter when used for the quantitative analysis of L and DCL separately.

REFERENCES

1 H. Davi, E. Marti, A. Bondon and W. Cautreels, in J. Rydström, J. Montelius and M. Bengtsson (Editors), Extrahepatic Drug Metabolism and Chemical Carcinogenesis, Elsevier, Amsterdam, 1983, p. 201.
2 J.P. Cano, Y.C. Sumirtapura, W. Cautreels and Y. Sales, J. Chromatogr., 226 (1981) 413.
3 H. Davi, J. Guyonnet, Y. Sales and W. Catureels, Arzneim.-Forsch., in press.
4 W. Cautreels and J.P. Jeanniot, Biomed. Mass Spectrom., 7 (1980) 565.