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Application of LC-MS analysis to the characterisation of the in vitro and in vivo metabolite profiles of RGH-1756 in the rat

L.I. Gémesi *, M. Kapás, Sz. Szeberényi

Gedeon Richter Ltd., 1103, Budapest Gyömrói út 19-21, Budapest, Hungary

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Abstract

RGH-1756, 1-(2-methoxy-phenyl)-4-{4-[4-(6-imidazol[2,1-b] thiazolyl)-phenoxy]-butyl-4-^{14}C}-piperazine dimethane is a novel atypical antipsychotic drug candidate of Gedeon Richter Ltd. The metabolic pathways of the compound have been investigated by profiling the metabolites present in plasma, bile, and faeces samples of rats treated with ^{14}C-RGH-1756. The metabolites formed in vitro by rat liver microsomes have also been analysed. Good separation of the compounds has been achieved by gradient HPLC method on Zorbax/Bonus RP-C18 column. Radiometry and mass spectrometry have been applied to detect and characterise the metabolites. The metabolite formed by oxidative cleavage of the chain at the carbon atom adjacent to the piperazine nitrogen has been identified as the major plasma metabolite. Glucuronide conjugate of hydroxy-RGH-1756 has been found as one of the main metabolites excreted in the bile where the unchanged compound has not been detected.

Keywords: RGH-1756; 1-(2-methoxy-phenyl)-4-{4-[4-(6-imidazol [2,1-b]thiazolyl)-phenoxy]-butyl-4-^{14}C}-piperazine; Antipsychotic; In vitro and in vivo metabolism; Rat; LC-MS; Turboionspray

1. Introduction

RGH-1756, 1-(2-methoxy-phenyl)-4-{4-[4-(6-imidazol [2,1-b]thiazolyl)-phenoxy]-butyl-4-^{14}C}-piperazine is a new drug candidate under preclinical development. The compound is a potent dopamine D3 receptor antagonist and may have potential as an atypical antipsychotic agent in the treatment of schizophrenia [1,2]. As part of drug safety evaluation studies, it was necessary to obtain early information on the metabolism of this compound as fast as possible. The objectives of this preliminary metabolism study were to profile and possibly identify the major metabolites present in plasma and excreta of rats following a single oral dose of ^{14}C-RGH-1756. Radiometry and mass spectrometry, the techniques typically applied for such studies, were used.

* Corresponding author. Tel./fax: +36-1-2605000.
E-mail address: larisza.gemesi@richter.hu (L.I. Gémesi).

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2. Experimental

2.1. Materials

Radiolabelled RGH-1756 (1-(2-methoxy-phenyl)-4-{4-[4-(6-imidazol [2,1-b]thiazolyl)-phenoxy]-butyl-4-^{14}C}-piperazine dimethane sulphonate) was synthesised at the Institute of Chemistry, Hungarian Academy of Sciences. Radiochemical purity was $>98\%$. Non-radiolabelled RGH-1756 (chemical purity: $>98\%$) and the chromatographic reference compounds AT 1458 (6-[4-(3-carboxy-propoxy)-phenyl]-imidazol [2,1-b]thiazole), AT 1450 (1-(2-hydroxy-phenyl)-4-{4-[4-(6-imidazol [2,1-b]thiazolyl)-phenoxy]-butyl-}-piperazine) and AT 1052 (6-(4-hydroxy-phenyl-imidazol[2,1-b]thiazole) were synthesised by Gedeon Richter Ltd. (Budapest, Hungary). Summary information on the reference standards are presented in Table 1.

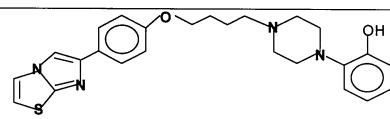
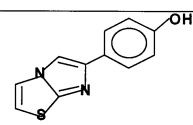
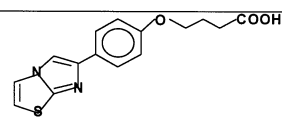
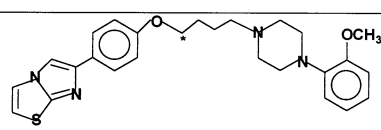
2.2. Microsomal incubation

Microsomal fractions were prepared from rat liver using published methods [3,4]. Reaction mixtures (2 ml) containing 260 μg RGH-1756, 10 mg liver microsomal protein, 1 mM nicotine amide adenine dinucleotide phosphate (NADPH), 5 mM glucose-6-phosphate, 0.5 IU/ml glucose-6-phosphate dehydrogenase, 4.2 mM $MgCl_2$ in 1.15% KCl–Tris–HCl buffer were incubated at 37°C under air for 60 min. The reaction was stopped by addition of 4 ml of acetonitrile. The precipitate was removed by centrifugation at 15 000 g for 10 min.

2.3. Animal dosing and sample collection

Thirteen male Wistar rats each received a single oral dose of 20 mg (4.75 MBq)/kg of [^{14}C]- RGH-1756 dissolved in distilled water. Blood was obtained by exsanguination of 4 animals at 1 h following drug dosing. The samples were cooled

Table 1
Summary information on reference standards[a]

Code	Structure	Molecular mass	Retention time (min)
AT 1450		448	37.8
AT 1052		216	24.5
AT 1458		302	23.2
RGH-1756		462	38.3

[a] Position of the [^{14}C] label.

on ice prior to centrifugation at 2000*g*, 4°C for 20 min. Bile samples were collected on ice from 4 animals during 0–6 h interval post-dose. Faeces samples were collected from 5 animals during 0–24 h interval post-dose and were homogenised after collection in a solution of methanol-distilled water (1:1). The samples of the individual animals were pooled and stored at −20°C until analysis.

2.4. Sample preparation

2.4.1. Precipitation of plasma and bile samples

Plasma and bile samples were subjected to protein precipitation with acetonitrile (1:1 and 1:3, respectively). The precipitate was then segregated by centrifugation at 15 000 g for 10 min and the supernatant was stored at −20°C until analysis.

2.4.2. Extraction of feacal samples

Feacal samples were extracted by methanol (1 g faeces homogenate + 8 ml methanol) and then centrifuged at 1000 g. The methanol extracts were concentrated to volume of 0.5 ml under nitrogen and stored at −20°C until analysis.

2.4.3. Solid phase extraction (SPE) of bile samples

Since simple protein precipitation of bile did not yield sufficiently clean extract for LC-MS analysis, SPE was applied using amino Baker-Bond cartridges (1 ml, 100 mg). The SPE cartridge was pre-conditioned with 0.5 ml of methanol followed by 0.5 ml of water. After sample loading (200 μl), the cartridge was rinsed with 0.5 ml of water and the retained components were eluted with 0.5 ml of 0.1 M ammonium acetate adjusted to pH = 3.5 with HCl. The eluates of 16 extractions were pooled and concentrated to a volume of 0.5 ml under nitrogen.

2.5. Metabolite profiling

2.5.1. LC-UV-Radiochemical analysis

Radio-HPLC profiles were obtained using HP 1050 HPLC system (Hewlett Packard) equipped with a quaternary pump, an auto sampler and a variable wavelength UV detector. A Radiometric 5000TR on-line radioactivity detector (Packard Cambera) with a liquid scintillator flow cell (0.5 ml) was fitted to the HPLC system.

Chromatographic separation was performed on Zorbax/Bonus RP-C18 column (150 × 4.6 mm, 5 μm). The mobile phases used were (A) water and 0.1 M ammonium acetate (90:10, v/v); (B) acetonitrile, methanol and 0.1 M ammonium acetate (60:30:10, v/v/v). The compounds were eluted using the following linear gradient: 0–10 min 80% A, 40 min 20% A, 40–60 min 20% A, 80 min 80% A. The flow rate was 0.6 ml min^{-1}, the column temperature was 40°C.

Unfortunately the peak resolution was compromised in the radiomonitor trace by the equipment configuration that was not optimal.

2.5.2. LC-MS analysis

The samples and reference compounds were analysed by an API 365 triple quadrupole LC-MS/MS mass spectrometer (Perkin Elmer Sciex Instruments) operating in single MS mode. The mobile phase was split 1:1 before the TurboIonspray (TIS) interface.

In the MS mode (full scan) the mass spectrometer scanned in the range m/z 150–700 with a dwell time of 0.5 ms and a step size of 0.1 amu. Turboionspray was operated in both positive and negative ionisation modes, where appropriate. The TIS voltages were set to 4100 and −4100 V, respectively. Temperature of heater gas nitrogen was 450°C. Ionisation conditions were optimised by infusing the available reference standards.

The chromatographic conditions were the same as for LC-UV-Radiochemical analysis. It is to be noted though, that there was a time shift (ca. 1 min) between the signal of the radio- and MS-detector due to the different equipment configurations.

3. Results

3.1. In vitro metabolite profile

The in vitro metabolite profile of RGH-1756 is presented in Fig. 1 and Table 2. Analysis of the mass spectral data has revealed that the metabo-

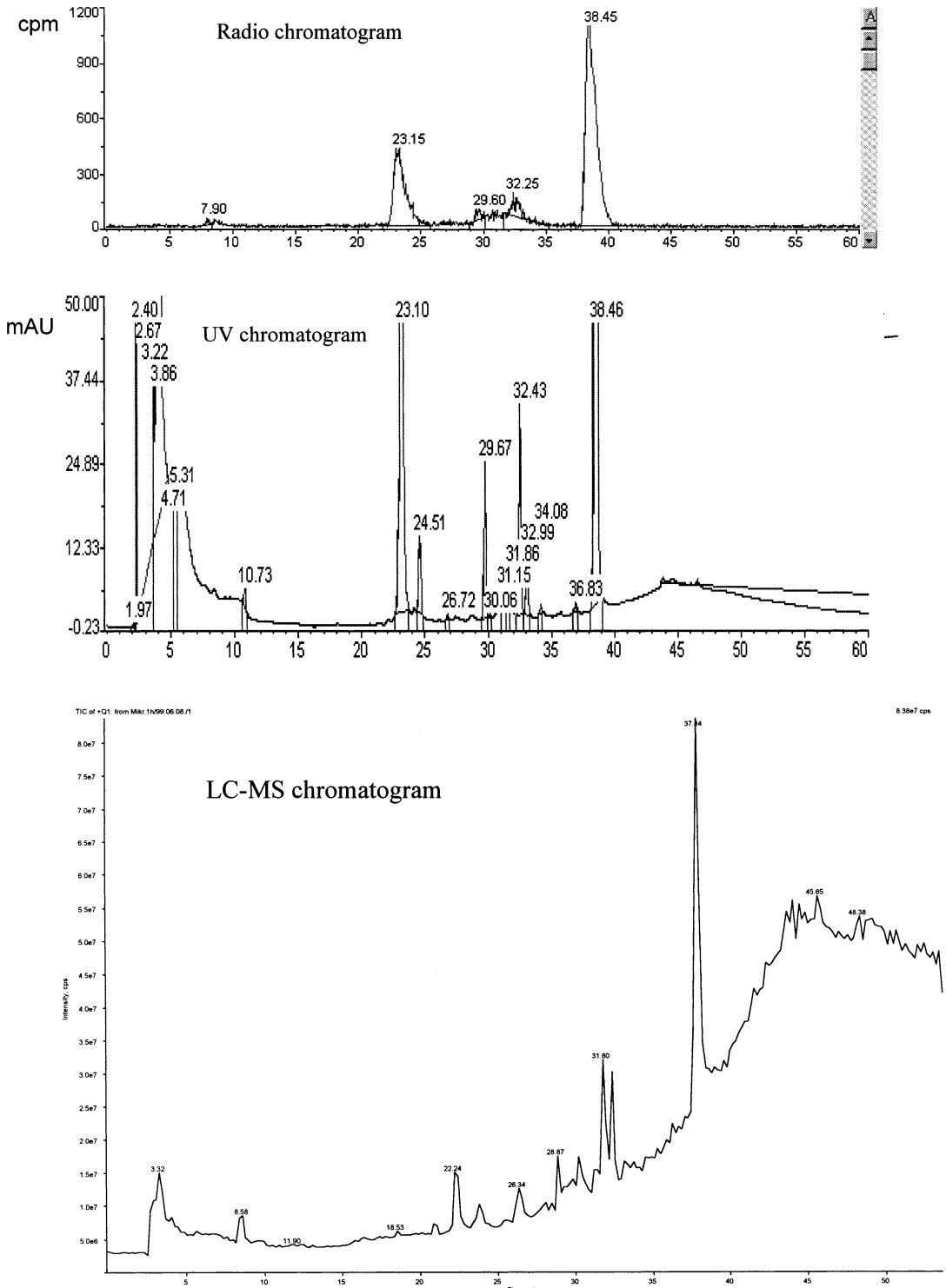


Fig. 1. Radio, UV and total ion chromatograms (TIC) of the extracts from rat liver microsomes. The difference in retention times stems from the use of different instruments.

Table 2
Summary information on the metabolites present in extracts of plasma, bile, faeces and microsomal incubate

Retention time (UV detection) (min)	Molecular mass	Microsomal incubation	Plasma	Bile	Faeces	Proposed identity
8.2	$M=278$	+[b]				Unknown
23.1	$M=302$	+	+			AT 1458
24.5	$M=216$	+				AT 1052
24.7	$M=654$			+		Hydroxy-RGH1756 glucuronide
26.7	$M=494$ ($M+32$)	+				Dihydroxy -or Hydroxy –sulphoxy –RGH-1756
29.4	Unknown			+		Unknown
29.7	$M=288$	+				Product of oxidative cleavage[a]
31.2	$M=478$ ($M+16$)	+				Hydroxy- or sulphoxy –RGH1756
32.4	$M=478$ ($M+16$)	+				Hydroxy- or sulphoxy –RGH1756
33.0	$M=478$ ($M+16$)	+				Hydroxy- or sulphoxy –RGH1756
37.8	$M=448$ ($M-14$)	+				AT 1450
38.4	$M=462$	+	+			RGH-1756

[a] 4-(4-Imidazo[2,1-b]thiazol-6-yl-phenoxy)-butan-1-ol.
[b] Detected.

lite formed in the highest quantity in vitro was an oxidative cleavage product of the parent drug. It co-chromatographed with the authentic standard AT 1458 ($R_t = 23.2$ min), and its molecular mass was confirmed by LC-mass spectrometry ($M = 302$). Three compounds with retention times between 31.2 and 33.0 min had the same MH^+ at m/z 479 (463 + 16) suggesting hydroxylation and/or sulphoxydation of RGH-1756. Mass spectrum of the metabolite eluting at $R_t = 26.7$ min showed addition of 32 amu (MH^+ at m/z 495), which indicates the presence of a dihydroxy- or hydroxy-sulphoxy RGH-1756 metabolite. The radioactive metabolite eluting with retention time 37.8 min was formed by *O*-demethylation. Its identity with the authentic standard AT 1450 was confirmed by molecular weight information gained from the LC/MS analysis ($M = 448$) and co-chromatography with the standard ($R_t = 37.8$ min). The non-radioactive metabolite with retention time 24.5 min co-chromatographed with the authentic standard AT 1052. TIC mass spectrum of the standard and the metabolite showed the same molecule ion MH^+ at m/z 217.

3.2. Plasma metabolites

Radiochromatogram of the plasma extract is shown on Fig. 2, with comparison to the profile obtained from microsomal incubate. The total ion chromatogram (TIC) of the extract is presented in Fig. 3. The radiochromatogram showed only one major peak, which co-chromatographed with the authentic standard AT 1458. The identity of the metabolite with the standard was confirmed by Full-scan LC-MS analysis in both positive (MH^+ at m/z 303) and negative (MH^- at m/z 301) ion mode. The radioactivity of this major peak amounted to 91% of the injected plasma radioactivity.

Unchanged RGH-1756 was also detected in the plasma extract ($R_t = 38.4$ min), representing about 3% of total plasma radioactivity. Its presence was confirmed by LC-MS analysis (MH^+ at m/z 463).

3.3. Bile metabolites

Sensitivity and resolution of the on-line radiodetector allowed to detect only 2 major metabolites in the bile extract (Fig. 2). The ra-

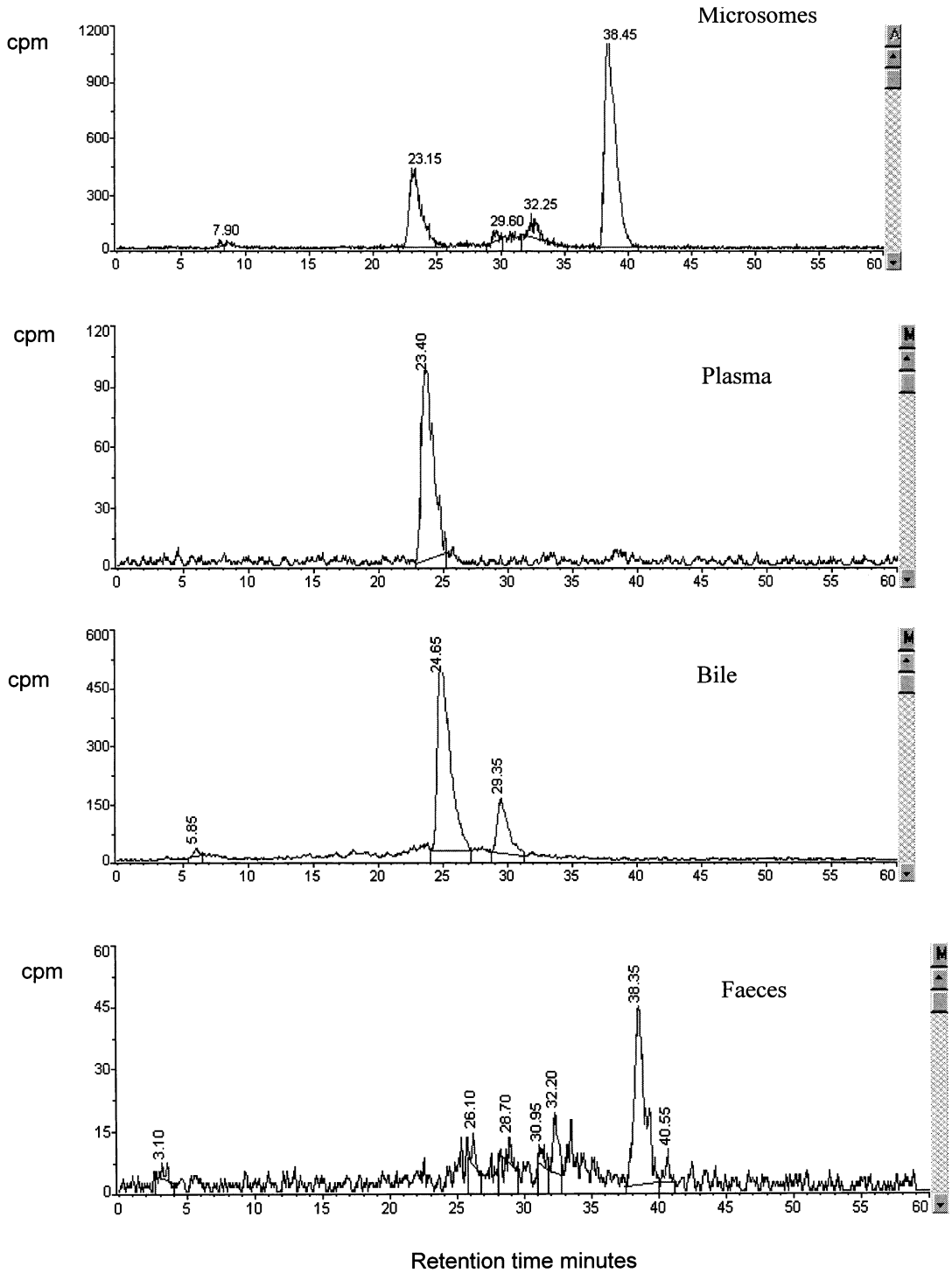


Fig. 2. Profiles of radioactivity in microsomal, plasma, bile and faeces extracts.

dioactivity related to these major metabolites was 65% ($R_t = 24.7$ min) and 16% ($R_t = 29.4$ min) of total bile radioactivity. Unchanged RGH-1756 was not found in this excretum. The metabolite with $R_t = 24.7$ min was identified by LC-MS (Fig. 3) as glucuronide conjugate of hydroxy-RGH-1756 (MH^+ at m/z 655). The molecular mass of the metabolite with $R_t = 29.4$ min could not been determined due to interference with endogenous compounds.

3.4. Faecal metabolites

The radioactivity profile of the faecal extract showed one major and some minor metabolites (Fig. 2). The major component co-chromatographed with the authentic RGH-1756 standard ($R_t = 38.3$ min) indicating that the unchanged compound was present in the faeces in a significant amount.

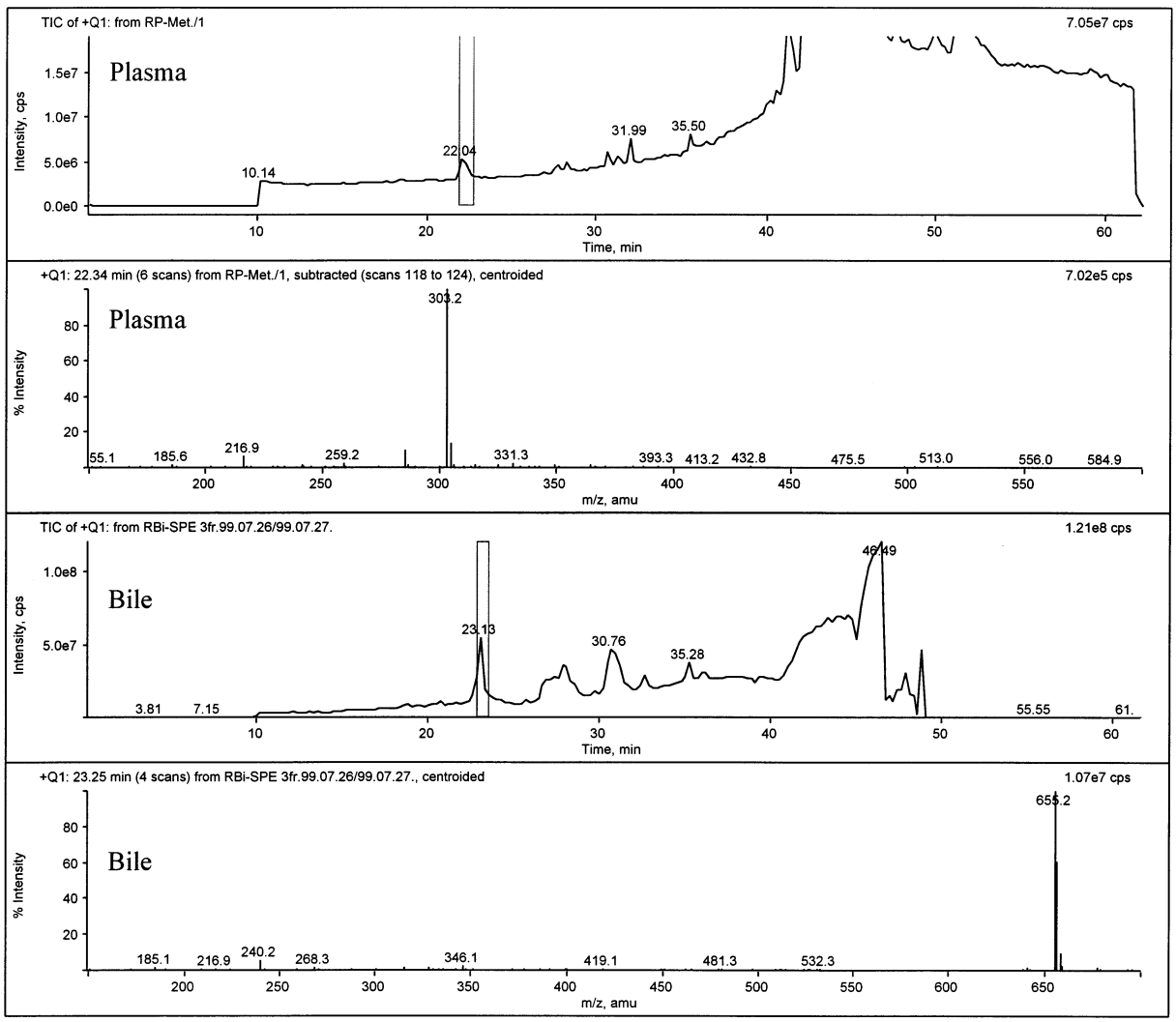


Fig. 3. Total ion chromatograms (TIC) and mass spectra of selected peaks following the analysis of plasma and bile extracts.

4. Discussion

In drug development it is very important that information on the metabolism of a drug candidate, i.e. on the nature and abundance of the metabolites, are available as soon as possible. Experimental data on the molecular weights can provide early confirmation of the theoretical assumption made a priori on the metabolic pathways and hence can trigger the early synthesis of the most probable standards.

Single LC/MS analysis is the first and ideal approach for molecular weight confirmation because in this mode virtually all the ion current is consolidated into an adduct of the molecular ion (i.e. $[M+H]^+$, $[M+NH_3]^+$) [5]. With regards to that the success of this method relies on the performance of the LC/MS interface and the ability to generate abundant ions that correspond to the molecular weight of the drug and drug metabolites, the ionisation conditions were thoroughly optimised so that the full scan mass spectra of the RGH-1756 standard would contain an abundant $[M+H]^+$ ion signal with no detectable fragmentation. TIS ionisation was chosen in preference to APCI in order to detect any potential phase II conjugates (e.g. glucuronides or sulphates), because the thermal instability associated with this class of metabolites often renders it difficult to obtain molecular weight information using APCI [6].

Detecting possible metabolites of a compound in a microsomal sample extract is usually less challenging than from a more complex matrix such as plasma, bile urine or faeces. Therefore the in vitro samples were investigated first and then, as a second step, the in vivo sample extracts were searched for previously identified molecular masses.

It was known from the mass balance study that 14C-RGH-1756 was excreted almost exclusively in the faeces and only traces of radioactivity (less than 3% of the dose) was found in the urine. Therefore the metabolism study was focused on profiling bile, faeces and plasma extracts. Correct interpretation of the metabolite profiles found in the various sample extract is not possible without taking into account of the extraction efficiency of the sample preparation. Recovery of radioactivity in the samples after deproteinisation was $\geq 80\%$, hence the radioactivity profiles presented for microsomal, plasma and bile samples (Fig. 2) can be considered as representative for the entire sample. Faeces is an exception because the recovery of radioactivity from the faeces sample by solvent extraction was ca. 50%. The less than complete recovery for the plasma (80%) might be explained by the strong binding of RGH-1756 to the plasma proteins (96%, unpublished data).

With the application of the techniques and approach described above the main pathways of the metabolism of RGH-1756 was successfully explored in a short period of time. The oxidative cleavage product, identified as 6-[4-(3-carboxy-propoxy)-phenyl]-imidazol[2,1-b]thiazole ($M = 302$) was the major metabolite of the compound in the plasma. Unchanged RGH-1756 was present in a small amount. The major metabolite in the bile was the glucuronide conjugate of hydroxy-RGH-1756. The low levels of RGH-1756 in plasma [7] and the absence of unchanged RGH-1756 in bile indicate that RGH-1756 undergoes extensive metabolism. Since unchanged RGH-1756 was absent in bile while high amounts of the parent compound were found in faeces (after radiolabelled oral dose) incomplete absorption of the drug can be assumed.

Acknowledgements

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References

[1] I. Laszlovszky, K.J. Kovács, M. Csejtjei, Naunyn-Schmiederberg's Arch. Pharmacol. Suppl. 1 to vol. 358 No1 (1998), p. 35. 122.
[2] B. Kiss, I. Laszlovszky, F. Auth, Naunyn-Schmiederberg's Arch. Pharmacol. Suppl. 1 to vol. 358 No1 (1998) p. 35. 124.
[3] D.E. Amacher, S.J. Shomaker, Toxicol. Lett. 94 (1998) 115–125.

[4] E. Larson, B. Howlett, A. Jagendorf, Analyt. Biochem. 155 (1986) 243–248.
[5] M.S. Lee, E.H. Kerns, Mass Spectrom. Rev. 18 (1999) 187–279.
[6] G.J. Dear, J.C. Harrelson, A.E. Jones, T.E. Johnson, S. Pleasance, Rapid Commun. Mass Spectrom. 9 (1995) 1457–1464.
[7] E. Terjéki, I. Laszlovszky, B. Kiss, M. Varga, É. Iván, M. Sönfeld M. Kapás, Naunyn-Schmiederberg's Arch. Pharmacol. Suppl. 1 to vol. 358 No1 (1998), p. 2. 10.