Integrated Analytical Strategies for Drug Discovery

de Vlieger, J.S.B.

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Chapter 2

High temperature liquid chromatography hyphenated with ESI-MS and ICP-MS detection for the structural characterization and quantification of halogen containing drug metabolites.

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Abstract

In this paper we describe the hyphenation of high temperature liquid chromatography with ICP-MS and ESI-MS for the characterization of halogen containing drug metabolites. The use of temperature gradients up to 200 °C enabled the separation of metabolites with low organic modifier content. This specific property allowed the use of detection methods that suffer from (significant) changes in analyte response factors as a function of the organic modifier content such as ICP-MS. Metabolites of two kinase inhibitors (SB-203580-Iodo and MAPK inhibitor VIII) produced by bacterial cytochrome P450 BM3 mutants and human liver microsomes were identified based on high resolution MS^n data. Quantification was done using their normalized and elemental specific response in the ICP-MS. The importance of these kinds of quantification strategies is stressed by the observation that the difference of the position of one oxygen atom in a structure can greatly affect its response in ESI-MS and UV detection.

1. Introduction

In early drug discovery programs, so-called metabolic stability assessment of drug candidates, it is important to predict the clearance of a drug by cytochrome P450 (and other) enzymes in the human liver. Early *in vitro* metabolic stability screening provides the medicinal chemists with information on the sites in the lead structures where metabolism is likely to occur (the so-called metabolic soft spots) [1]. The identification and quantification of metabolites requires advanced analytical technologies and the development in this area is an ongoing process [2]. Recently, we introduced a platform for the simultaneous bioactivity testing and identification of drug metabolites [3]. Depending on the properties of the drugs investigated and the bioassay applied, the platform may be adapted to include alternative separation and/or detection technologies. One of the limitations of an on-line bioassay is the compatibility with LC mobile-phase composition [4], especially with respect to the organic modifier content. High-temperature liquid chromatography (HTLC) has been demonstrated to enable compound separation at constant (low) organic modifier content. Teutenberg *et al.* described the fundamentals and practical issues of HTLC in detail [5,6], including the use of low organic modifier concentrations. The benefits of HTLC combined with on-line

bioassays were readily demonstrated [7]. However, a constant organic modifier content is beneficial in other instances as well. Several analyte detection strategies suffer from (significant) changes in analyte response factors as a function of the organic modifier content. This may lead to under- or over-estimated concentrations of drug metabolites present in the sample. This is certainly true for attractive tools like evaporative light scattering detection (ELSD) [8,9], electrospray ionization mass spectrometry (ESI-MS) [10] and inductively coupled plasma-mass spectrometry (ICP-MS). In the latter case, a solvent gradient does not only influence the response factor but also the stability of the ICP plasma. HTLC-ICP-AES was recently reported for the analysis of food ingredients [11]. Smith *et al*. pioneered the integration of HTLC with ICP-MS [12] for the quantification of a glycine conjugate of bromobenzoic acid.

Since its development, ICP-MS is mainly used for quantitative trace element determination. The main application area of elemental speciation is found in environmental studies. In pharmaceutical applications, the use of ICP-MS started with the profiling of inorganic impurities and nowadays is expanding to bioanalysis as well, especially involving pharmacokinetic studies of platinum based cytostatics [13,14]. More recently, ICP-MS has to a limited extent been applied in drug metabolism studies of drugs readily detectable by ICP-MS, especially drugs containing Cl and Br, as reviewed by Gammelgaard *et al*. [15]. Although fluorine is an even more occurring halogen in drug molecules, low-level detection of fluorine by ICP-MS is hampered by its relatively high ionization energy. The elemental specificity, the large linear range, and its ability to quantify with an species-unspecific elemental standard are features of ICP-MS that can be used complementary to e.g. ESI-MS. Cuykens *et al.* reported the use of ICP-MS with isotope dilution for profiling a drug in development [16] as well as the use of ICP-MS for the detection of glutathione adducts of clozapine based on S and Cl [17]. Wilson *et al.* reported interesting work on metabolite detection in biological fluids with ICP-MS [18-20]. In this paper, we describe the application of HTLC in the identification and quantification of drug metabolites based on ESI-HR-MS and ICP-MS. Oxidation products of two kinase inhibitors, SB-203580-Iodo and MAPK inhibitor VIII, were generated with human liver microsomes, bacterial cytochrome P450 BM3 mutants and electrochemical oxidation.

Experimental

2.1. Materials

Human liver microsomes (HLM), pooled from 50 donors were obtained from Xenotech (Lot No. 0710619) and contained 20 mg/mL protein. Drug metabolising bacterial cytochrome P450 BM3 mutants M02 [21] and M11 V87A [22] were provided by dr. J.N.M. Commandeur (section Molecular Toxicology, VU University Amsterdam). Acetonitrile (ACN), methanol (MeOH) both LC-MS grade and formic acid (F) ULC-MS grade were obtained from Biosolve (Valkenswaard, the Netherlands). Purified water was produced by a Milli-Q device of Millipore (Amsterdam, the Netherlands). Applichem (Lokeren, Belgium) supplied β-nicotinamide adenine dinucleotide phosphate (NADPH) tetra sodium salt. Isoclozapine was obtained from Axon Medchem (Groningen, the Netherlands), Clothiapine from Enzo Life Sciences (Raamsdonkveer, the Netherlands) and loxapine from Sigma Aldrich (Zwijndrecht, the Netherlands). SB-203580-Iodo (SB-I) and p38 MAP kinase inhibitor VIII were obtained from Calbiochem (Amsterdam, the Netherlands). Monosodium dihydrogen phosphate, disodium monohydrogen phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, ammonium acetate and dimethylsulfoxide (DMSO) were purchased at Sigma-Aldrich (Schnelldorf, Germany). The structures of all analytes are provided in Figure S1 (Supplementary information).

2.2. Methods

Metabolic incubations with the P450 BM3 mutants had a final volume of 500 µL and consisted of 500 nM enzyme and 100 µM substrate in 100 mM potassium phosphate buffer pH 7.4 and were performed at 24 °C. Of the substrates, stock solutions were made of 10 mM in DMSO. The DMSO concentration in the metabolic incubations was always below 2 %. An NADPH regenerating system was used to initiate the reactions, resulting in final concentrations of 0.2 mM NADPH, 0.3 mM glucose-6-phosphate, and 0.4 units/mL glucose-6-phosphate dehydrogenase.

Metabolite generation with HLM was performed at 37 °C. The incubations had a final volume of 500 µL and consisted of 1 mg/mL protein and 100 µM substrate in 100 mM potassium phosphate buffer pH 7.4 containing 5 mM $MgCl_2$. At time points 0 min and 90 minutes, 250 µL samples were taken from the incubation and

added to 250 µL of ice-cold ACN, vortexed for 30 seconds and centrifuged for 15 minutes at 6000 rpm. The supernatants were filtered through a Phenex RC syringe filter 0.2 µm from Phenomenex (Utrecht, the Netherlands).
Electrochemical oxidation of SB-I was done by infusing a 10 µM standard in 25% ACN, and 1 mM ammonium acetate buffer with pH 5.0 through a Roxy reactor cell equipped with a glassy carbon electrode controlled by a Decade II module and Dialogue software (Antec Leyden, Zoeterwoude, the Netherlands). Products were formed at +1.5 V with a flow-rate of 10 µL/min and collected in an autosampler vial insert.

2.2.1. HTLC-ESI-MS

A Shimadzu LC Ion Trap – Time of Flight (IT-TOF) MS system (Shimadzu, 's Hertogenbosch, the Netherlands) was equipped with an HT-HPLC 200 oven from SIM GmbH (Oberhausen, Germany). A Waters Acquity UPLC BEH C18 1.7 µm, 2.1 × 50 mm column was used for separation. The column was mounted inside the oven with an in house made aluminium holder for optimal heat transfer (see Figure 1 for schematic representation of the setup). A stainless steel frit (1 µm) fixed into a Valco union (DaVinci Europe, Rotterdam, the Netherlands) was used as an inline filter. The integrated solvent preheating area and post-column cooling unit consisted of 25 cm and 30 cm stainless steel tubing (internal diameter of 0.3 mm). In all HTLC experiments, the solvent preheating area was set 5 °C above the starting temperature of the gradient while the post column cooling unit was set at a fixed value of 25 °C.

The IT-TOF-MS was equipped with an electrospray source and operated in positive ion mode. The interface voltage was set at 4.5 kV while the source heating block and curved desolvation line (CDL) temperature were at 200 ˚C. Drying gas pressure was set at 62 kPa and nebulising gas was applied. MS^3 spectra were acquired in data dependent mode with the following settings: full-spectrum MS from *m/z* 200 – 650, MS^2 and MS^3 acquisition from *m/z* 80-650 with ion accumulation of 10 ms and collision energy set at 50 %. The first minute of the chromatogram was diverted to waste.

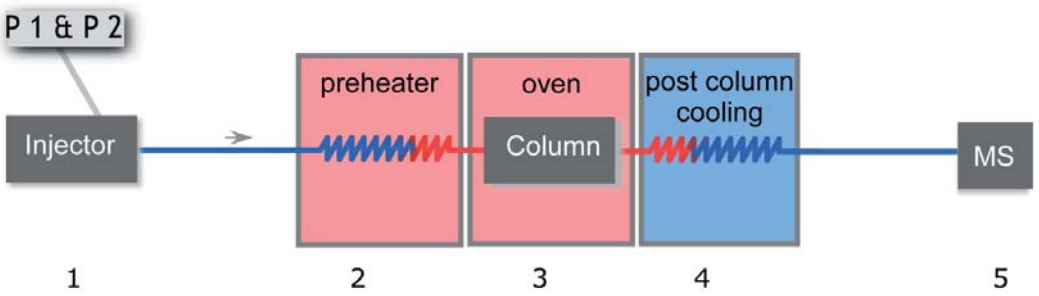

Figure 1: Schematic representation of the HTLC-MS setup with, (1) Injector and gradient pumps, (2) Solvent preheating compartment, (3) Column heating block, (4) Post column cooling unit and (5) ICP-MS or ESI-MS.

A standard mixture of 100 µM isoclozapine, clothiapine and loxapine in 3% DMSO, 97% H_2O was analysed in isocratic mode, with eluent composition of 95% H_2O, 0.2% FA and 5% ACN or 5% MeOH (solvent Aa and Am, respectively). If not stated otherwise, a flow-rate of 300 µL/min was applied. With 5% ACN as eluent, the temperature gradient started with 0.5 minute at 110 °C followed by a linear increase up to 175 °C in 6 minutes where it was kept constant for 2.5 minutes. With 5% MeOH as eluent, the temperature gradient started with 0.5 minutes at 130 °C followed by a linear increase up to 175 °C in 4 minutes where it was kept constant for 2.5 minutes. The system was ready for the next injection after 12.5 minutes. For reference chromatograms, the following solvent gradient was applied: 1 minute isocratic at 5% B linear increase in 11.5 minutes to 75% B, back to 5% B in 0.5 minutes where kept constant for 3 minutes. Solvent Ba and Bm were ACN, 0.2% FA and MeOH, 0.2% FA, respectively. The column was thermostated at 50 °C.

The analysis of the SB-I samples was achieved using the following temperature gradient: 5 minutes at 110 °C, then a linear increase to 180 °C in 8 minutes where kept constant for another 5 minutes. The system was cooled down and after a total runtime of 20 minutes, it was ready for the next injection. An isocratic solvent Aa was used with a flow-rate of 600 µL/min.

MAPK inhibitor VIII samples were analysed with a temperature gradient starting from 120 °C for 5 minutes, followed by a linear increase in 5 minutes to 200 °C

where kept constant for 5 minutes. An isocratic solvent composition of 15% MeOH 0.2% FA was used with a flow-rate of 600 µL/min.

Thermostability of the standards was assessed by determining the peak areas of the standards in a set of experiments where isocratic and isothermal elution was performed. With increasing the temperature up to 200 °C, this experiment can indicate possible on-column degradation.

2.2.2. ICP-MS

An Agilent 7500c ICP-MS equipped with a collision/reaction cell (Agilent Technologies, Amstelveen, the Netherlands) was used for the ICP-MS analysis. Tuning of the instrument was done using direct infusion and pneumatic nebulisation of the analyte solution in the mobile phase corresponding to its isocratic elution parameters. The plasma parameters for Br and I detection were as follows: RF power 1600W, RF Matching 1.72 V, Sample depth 6.0 mm, Torch horizontal positioning 0.2 mm and vertical 0.6 mm. The carrier gas was 0.82 L/min and the makeup gas was set to 0.10 L/min, both gases were argon with 5.0 purity. For the detection of SO^+, the RF power was 1600 W, the RF matching 1.70 V, sample depth 12.0 mm, torch horizontal and vertical position were set at -0.4 mm. The carrier gas was 0.90 L/min and the makeup gas 0.20 L/min. The optional gas (O_2) was set at 5% and was added to the plasma. The transfer line between the HTLC oven and the inlet of the ICP-MS was 50 cm of PEEK tubing with an internal diameter of 0.005 inch.

3. Results and discussion.

Thermal degradation of the standards was not observed under the conditions used. In the supporting information (Figure S2) the extracted ion chromatograms of SB-I (as an example) are shown under increasing temperatures while isocratic elution was performed. The calculated peak areas had a standard deviation of less than 5 percent, indicating that the compound was stable throughout the analysis.

3.1. Solvent effect on electrospray ionization.

For the three standards, isoclozapine ($C_{18}H_{19}ClN_4$, $[M+H]^+$ with *m/z* 327.1371), loxapine ($C_{18}H_{18}ClN_3O$, $[M+H]^+$ with *m/z* 328.1211) and clothiapine ($C_{18}H_{18}ClN_3S$, $[M+H]^+$ with *m/z* 344.0983), a comparison was made between the peak area in

ESI-MS using either a temperature or a solvent gradient. When using a temperature gradient at 5% of organic modifier, the peak areas for the standards were affected by the type of organic modifier (MeOH or ACN) to a very small extend, as can be seen in Figure 2. In the solvent gradient, the peak areas in MeOH were higher than those in ACN (24% for isoclozapine, 17% for loxapine and 16% for clothiapine), which is somewhat to be expected because ACN is the stronger solvent, making the analytes elute at a lower ACN than MeOH concentration in the gradient. The solvent effect on the ESI-MS response obviously is a bit of a disadvantage in HTLC, where only 5% of organic modifier is used. Especially loxapine and clothiapine, eluting at higher concentrations of organic modifier in the solvent gradient, show a factor of 1.9 and 2.3, decreased ESI-MS peak areas in the temperature gradient (Figure 2), respectively. The chromatograms of using a temperature gradient and a solvent gradient with ACN can be found in the supporting information, Figure S3. In practical applications, not only the peak area but also the signal-to-noise ratio (S/N) is important. In the total-ion chromatogram (TIC), there is not much difference in S/N for the three compounds, when comparing the solvent and the temperature gradient. In the extracted-ion chromatograms (EIC), the S/N in HTLC mode is slightly lower than the S/N of the solvent gradient. This is consistent with the difference in ESI-MS response between the two modes as described above.

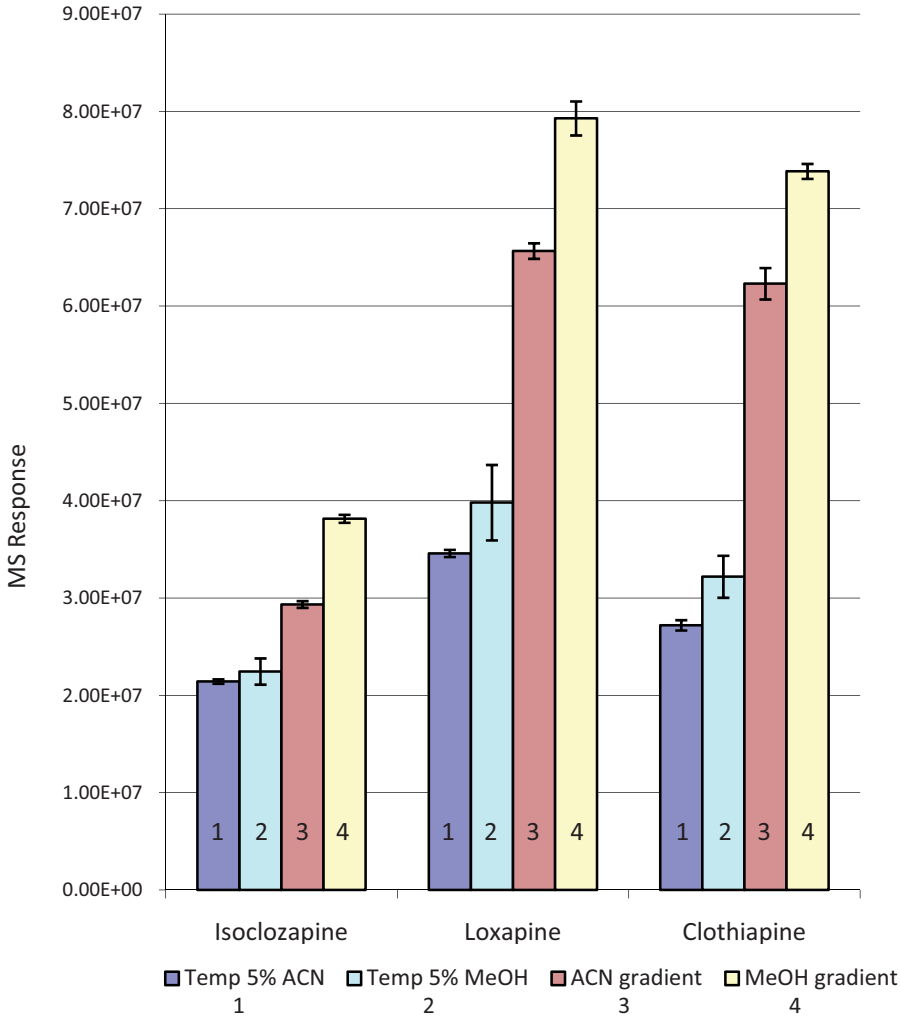

Figure 2: Comparison of ESI-MS peak areas (arbitrary units) of standards with temperature and organic solvent gradients.

3.2. Hyphenation of HTLC to ICP-MS.

The detection limits of the ICP-MS for Cl^+ (*m/z* 35), Br^+ (*m/z* 79), S^+ (*m/z* 32) and I^+ (*m/z* 127) were determined by flow injection in a 5 % MeOH or ACN, 0.2 % FA solution. Using optional gas flow, the detection of SO^+ (*m/z* 48, LOD 18 µM) was found to be more sensitive than that of S^+ (227 µM). This is due to the occurrence of a polyatomic interference of O_2^+ (m/z 31.9898) with S^+ (*m/z* 31.9721), both having *m/z* 32 in the ICP-MS due to its limited resolution. Isoclozapine, loxapine

and clothiapine were readily separated and detected with the HTLC-ICP-MS method. The chlorine specific detection demonstrated the hyphenation of HTLC to ICP-MS, with an LOD of 128 µM. As can be seen in Figure 3, the chromatogram of *m/z* 35 of the HTLC-ICP-MS analysis of a 250 µM mixture of clothiapine (5), iso-clozapine (3) and loxapine (4) resulted in five peaks with an S/N ratio above 3.

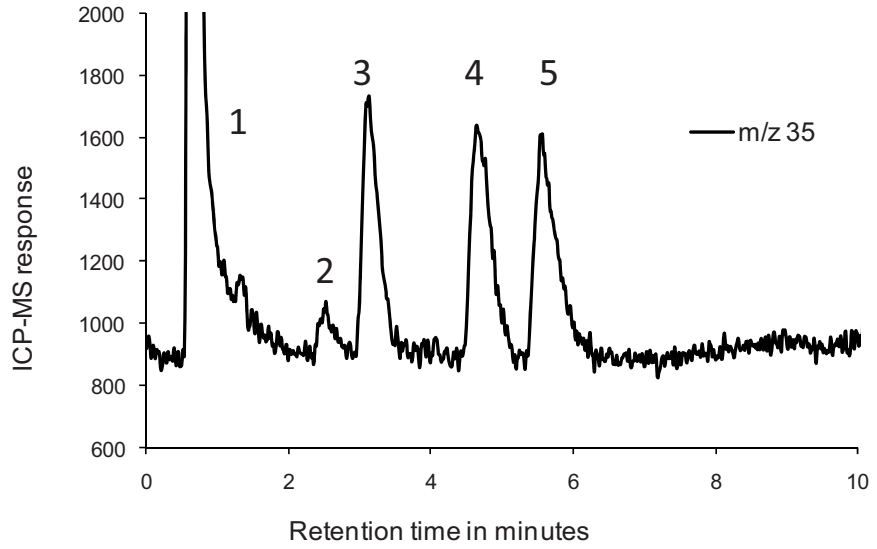

Figure 3: HTLC-ICP-MS chromatogram for chlorine (*m/z* 35) in a 250 µM standard mix of iso-clozapine, loxapine and clothiapine using 5% ACN 0.2 % FA as mobile phase. (1) polyatomic interference of $^{34}S+H$; (2) Chlorine containing impurity with m/z 355.1325; (3) isoclozapine; (4) loxapine; (5) clothiapine.

Peak number 1 is due to polyatomic interference of $^{34}S+H^+$ (*m/z* 35) resulting from the high percentage of DMSO in the sample, or to the breakthrough of $^{35}Cl^+$. (The unit-mass resolution ICP-MS used in this study does not allow discrimination of these two.) Peak 2 is due to an impurity in the isoclozapine standard. HR-MS data indicate an $[M+H]^+$ with *m/z* 355.1325 and a monochlorine isotope pattern for this impurity. The molecular formula is $C_{19}H_{19}ClN_4O$, corresponding to additional CO in the molecule. Unfortunately, in MS/MS of the impurity, only the loss of H_2O is observed. Therefore, it is difficult to make a structure proposal. Peaks 3, 4, and 5 were identified based on the HR-MS data as isoclozapine, loxapine and clothiapine, respectively.

For bromine with *m/z* of 79, the detection limit in ICP-MS was 0.5 µM and iodine could be detected down to 80 nM. For iodine, a linear calibration plot was achieved in the range of 80 nM to 80 µM (y = 3.96e4 x + 2.17e3 with R^2 0.9976), the experimental conditions of the HTLC part of this are described above in section 2.2.1. The detection limits were not as low as reported by others [15], but are sufficiently low for the detection of metabolites and impurities present in the *in vitro* incubation samples. Moreover, the separation method differs significantly and our injection volumes are a factor of 2 to 10 smaller.

3.3. Profiling bromine containing MAPK inhibitor VIII products.

The ESI-MS^n fragmentation pattern of MAPK inhibitor VIII ($[M+H]^+$ with *m/z* 415.0207, $C_{20}H_{17}BrClN_2O^+$) in the IT-TOF was elucidated to facilitate the identification of its metabolites. Figure 4a shows the ESI-MS^n spectra and Figure 4b the proposed fragmentation pathway.

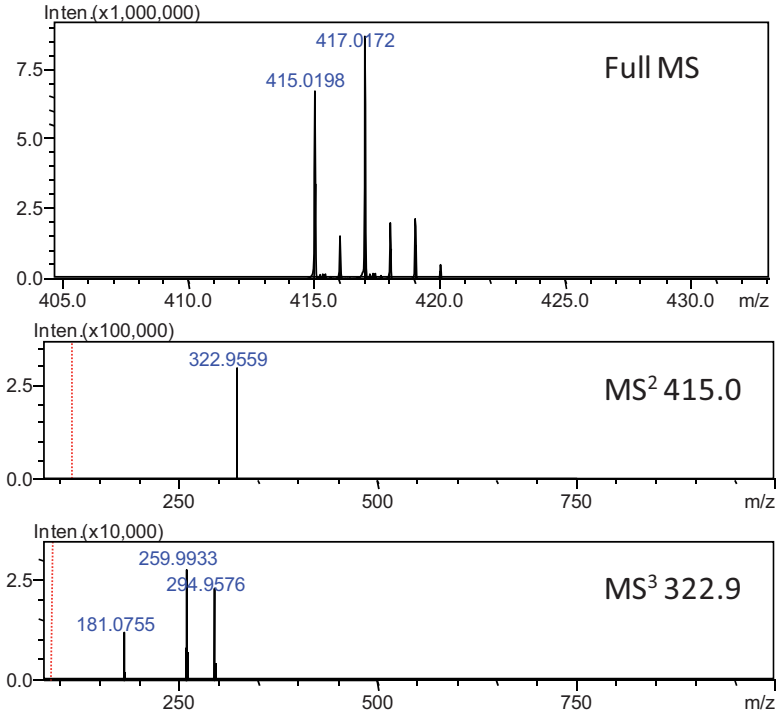

Figure 4a: Full HR-ESI-MS, MS/MS and MS^3 spectra on MAPK inhibitor VIII.

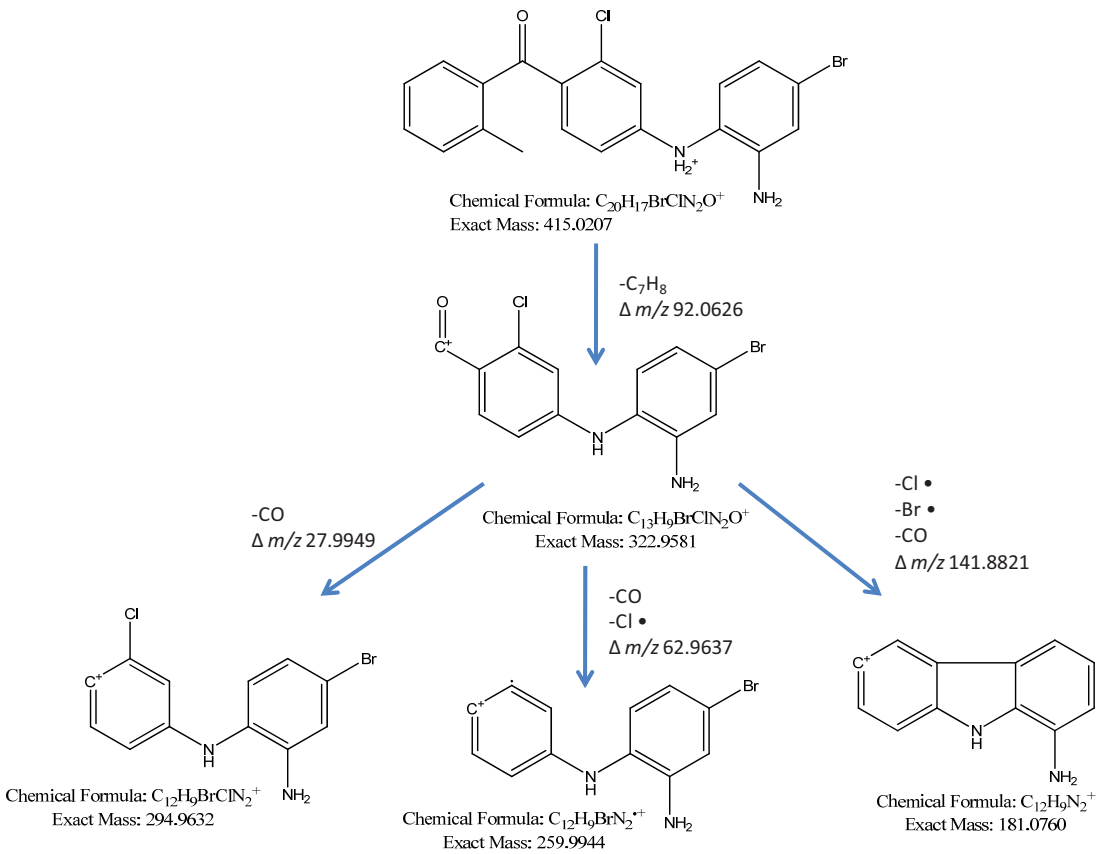

Figure 4b: Proposed fragmentation pattern and fragment structures of MAPK inhibitor VIII.

MAPK inhibitor VIII contains both a chlorine and a bromine atom; the characteristic isotopic pattern is observed in ESI-MS. This feature is useful for the search of structurally related metabolites in the incubation mixture by e.g. software assisted isotope pattern recognition and isotope filtering strategies [23]. In this case, we used HTLC-ICP-MS to search for all bromine containing compounds in the metabolic incubations. Subsequently, these bromine containing metabolites were identified based on their MS^n spectra in the ESI-MS measurements.

Two metabolites of MAPK inhibitor VIII were found in the P450 BM3 mutant incubation samples when analysed with HTLC-ICP-MS (Figure 5) and LC-ESI-MS. The accurate masses of these metabolites are in accordance with a hydroxylated

substrate ($[M+H]^+$ with *m/z* 431.0156, peak 2 in Figure 5) and a quinone-type structure ($[M+H]^+$ with *m/z* 444.9949, peak 1 in Figure 5). The fragmentation patterns of these metabolites indicate that the oxidations took place in the toluene substructure, present in MAPK inhibitor VIII.

The singly oxygenated metabolite (peak 2) could be tentatively identified from a different fragmentation behaviour in MS^n compared to the parent drug. It showed a loss of H_2O in MS^2 and subsequent loss of a bromine radical in MS^3, and not the toluene loss observed for the parent drug. Therefore, we propose that hydroxylation occurred at the methyl group and the water loss involves a six-member ring formation between the bromo-phenyl group and the chlorine substituted benzene ring. The loss of a halogen radical from an aromatic systems is often observed in ESI-MS/MS experiments [24]. The other metabolite (peak 1) shows a mass difference of 29.9731 u, which is consistent with the loss of H_2 and the gain of O_2 (29,9742 u), thus $C_{20}H_{15}BrClN_2O_3^+$. In MS^2, this metabolite shows the fragment ion with *m/z* 296.9789, indicating the loss of the (modified) toluene ring. The most likely way to explain the observed changes in the toluene ring is by quinone formation [25]. This apparently results in an easier loss of the complete side chain ($C_8H_4O_3$) with hydrogen rearrangement rather than the stepwise loss of toluene and CO, as observed for the parent compound.

The HTLC-ICP-MS chromatogram showed an additional bromine containing compound in the sample (peak 3, Figure 5), and was found to be an impurity in the analytical standard (*m/z* 439.0192, proposed formula $C_{22}H_{17}N_2OClBr^+$, 4.8 ppm error). The acquired MS^2 data allowed us to propose a structure for this impurity (Figure 5). The formation of a six membered ring by adding C=C to connect the secondary substituted amine with the primary amine fits the accurate mass and matches the fragmentation spectra. Peak 4 is the substrate, MAPK inhibitor VIII with *m/z* 415.0207. In the HTLC-ESI-MS chromatograms, we did not observe additional metabolites or impurities missing the characteristic bromine and/or chlorine isotope pattern of MAPK inhibitor VIII.

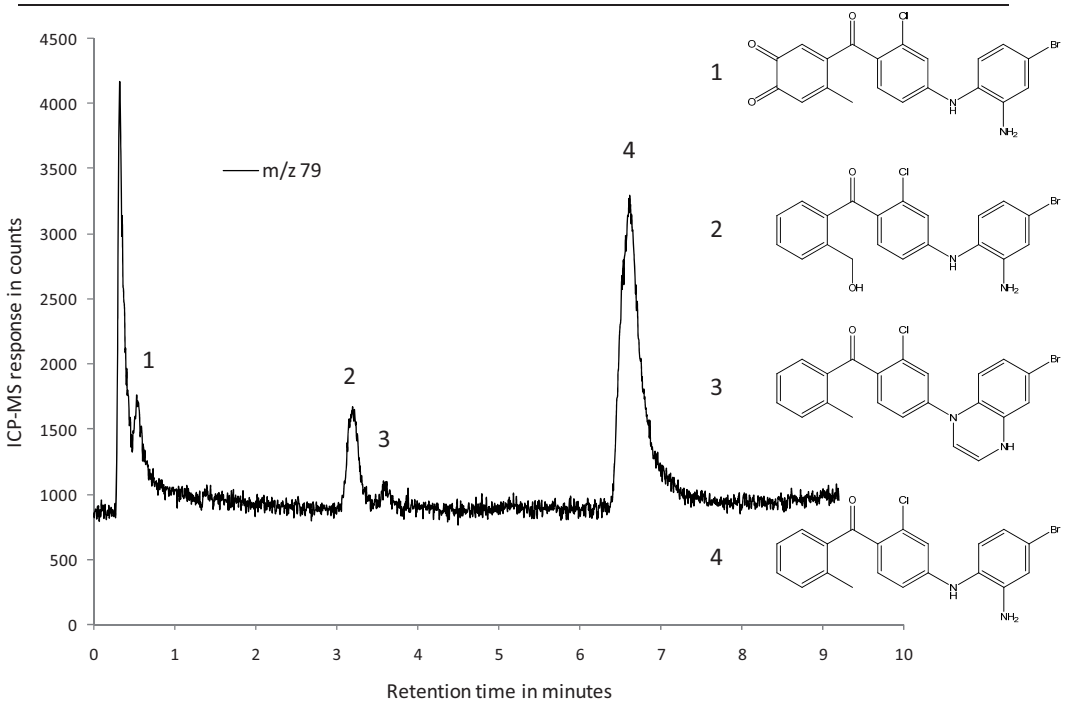

Figure 5: HTLC-ICP-MS chromatogram (m/z 79, Br^+) of the incubation sample having MAPK inhibitor VIII as substrate.

3.4. Analysis of iodine containing SB-I and conversion products.

The major fragment in MS^2 of SB-I ($C_{21}H_{17}IN_3OS^+$, $[M+H]^+$ with *m/z* 486.0132) in the IT-TOF instrument is the loss of a methyl radical, resulting in the fragment with *m/z* 470.9894. Performing MS^3 experiments on this radical cation produces a major fragment with *m/z* 423.0227 corresponding to the loss of SO. In addition, a minor fragment with *m/z* 344.0843 is observed due to the loss of an iodine radical. The limited fragmentation under these conditions indicates that unambiguous metabolite identification by MS^n will not be readily possible. Incubation of SB-I with the P450 BM3 mutants M02, M11 V87A and with HLM resulted in the formation of several metabolites (see Figure 6). This figure may be used to assess the ease at which HTLC is implemented: ESI-MS^n and ICP-MS were performed on different days and in two different laboratories (Amsterdam and Groningen). In order to achieve this, the HTLC part of the system was dismantled in the first and reinstalled in the second laboratory. Nevertheless, highly comparable chromatographic results were achieved (Figure 6).

MS^n spectra obtained by HTLC-ESI-MS^n analysis of the metabolites of SB-I, indicate oxidation at the sulphur, oxidation of the dehalogenated SB-I (*m/z* 376.1144) and two more oxidation products (*m/z* 502.0088). S-oxidation has also been reported for the fluorine substituted analogue SB-203580 [26]. Dehalogenation of the standard (*m/z* 360.1181) was observed in all samples, including the controls and dilutions from a freshly prepared stock solution.

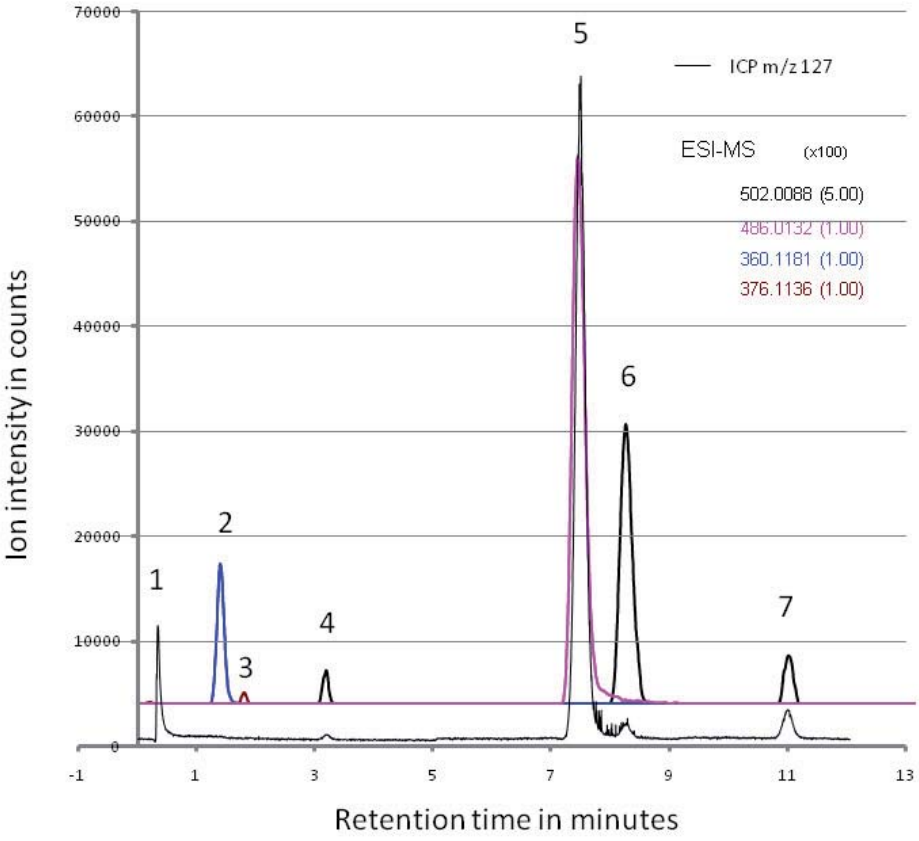

Figure 6: Comparison of HTLC-ICP-MS chromatogram with m/z 127 (lower trace) and HTLC-ESI-MS chromatogram with extracted ions of SB-I and related products. (m/z 502.0088 5x magnified)

Peak 1 in the ICP-MS chromatogram (figure 6) is observed in the enzyme blank and in the t=0 control as well, indicating a background signal of the human liver microsomal matrix and is therefore not included in table 1. Peak 2 in the ESI-MS chromatogram with *m/z* 360.1181 can be identified as the dehalogenated SB-I and is present as an impurity in all samples containing SB-I. Increased ESI-MS peak

areas in the active incubations compared to control experiments indicate that dehalogenation is catalysed by the P450s. Since iodine is no longer present in the structure, this compound is not detected in the ICP-MS analysis. The presence of sulphur provides an additional feature to quantify these metabolites, although we were able to detect S^+ and SO^+, these detection limits were not sufficiently low enough to be used for quantification of these low abundant metabolites. Peak 3 is a metabolite present in all active enzymatic incubations but the P450 BM3 mutants produce significantly more of this oxidized dehalogenated metabolite (*m/z* 376.1136, see table 1). The loss of a CH_3SO_2 radical indicates oxidation of the sulphur. There are two possible explanations for the formation of this metabolite, either oxidation of the dehalogenated impurity or the reductive dehalogenation of the main metabolite, the sulphonated SB-I. More interesting is peak 4, present in both ICP-MS and ESI-MS traces of the HLM incubation. This indicates the presence of iodine in the structure. The *m/z* value is in accordance with hydroxylation of the parent compound (*m/z* 502.0088). This metabolite is only found in the HLM incubations and not in the mutant ones. Peak 5 is the parent compound with *m/z* 486.0132. Based on the ESI-MS data, the main metabolite was found to be oxidized at the sulphur into a sulphone, peak number 6, as is indicated by the subsequent losses of a CH_3 radical and SO_2 in two stages of MS^n. This metabolite was found in all incubations, but at different concentrations (table 1). In incubations with HLM and with the mutant M11 V87A only, an additional oxidated product with *m/z* 502.0088 was found (peak 7). Given the fact that the retention time for this product is higher than that of the parent, the compound might be an N-oxide [27]. With the formation of this specific compound, the M11 V87A BM3 mutant mimics the in vitro human metabolism the most.

Table 1: Formation of SB-203580-Iodo related products by different enzymatic systems including ICP-MS and ESI-MS data.

Peak nr	Rt in minutes	Enzyme	ICP-MS		ESI-MS		
			area	conc in µM	area	[M+H]+	Molecular formula
2	1.4	M02	-	-	15693726	360.1181	$C_{21}H_{17}N_3OS$
		M11 V87A	-	-	14731403		
		HLM	-	-	11823036		
3	1.8	M02	-	-	4046358	376.1136	$C_{21}H_{17}N_3O_2S$
		M11 V87A	-	-	3843774		
		HLM	-	-	582953		
4	3.2	M02	-	-	-	502.0088	$C_{21}H_{16}IN_3O_2S$
		M11 V87A	-	-	-		
		HLM	3999	<0.08 (0.05)	437422		
5	7.7	M02	917471	23.1	113167098	486.0132	$C_{21}H_{16}IN_3OS$
		M11 V87A	876649	22.1	104141596		
		HLM	746022	18.8	82157923		
6	8.4	M02	31814	0.75	25544346	502.0086	$C_{21}H_{16}IN_3O_2S$
		M11 V87A	20698	0.47	15789179		
		HLM	17126	0.38	8225095		
7	11.0	M02	-	-	-	502.0086	$C_{21}H_{16}IN_3O_2S$
		M11 V87A	27128	0.63	998657		
		HLM	30589	0.72	1044683		

3.5. Response comparison of ICP-MS and ESI-MS.

Table 1 not only provides information on the concentrations of metabolites, based on the response in ICP-MS, but also enables a comparison of the response factor in ESI-MS and ICP-MS. For example, the metabolite peaks 6 and 7 have the same chemical formula and the oxygen incorporated by metabolism results in only a relatively small difference in the chemical structure. However, whereas the concentrations of these metabolites are very similar (0.75 µM peak 6 and 0.72 µM

for peak 7), the response in ESI-MS differs by a factor of ~25. (25.5×10^6 for peak 6 and only 1.0×10^6 for peak 7). The ICP-MS is a normalized detector for specific elements and therefore not sensitive to the difference in structure of these two metabolites. Additionally, the results of comparing the same calibration samples measured on both instruments show the difference in linearity, especially with the higher concentrations of SB-I measured. Calibration curves were measured from 0 to 80 µM of SB-I. The LOD for SB-I with HTLC-ICP-MS was 80 nM while with ESI-MS the LOD was as low as 10 nM. The linear range of the ICP-MS was found to be from 80 nM up to 80 µM, while in HTLC-ESI-MS linearity was observed from 20 nM to 10 µM. This demonstrates the potential of over- and/or underestimating the concentration of a metabolite based on its response in a regular LC-ESI-MS run. In this case, the small structural changes in the molecule and the limited linear response in ESI-MS require complementary normalized detection methods to provide an accurate assessment of the concentrations present in the sample.

Next to incubations with enzymes, we apply electrochemical oxidation to generate oxidation products of drugs. In some cases, several human relevant metabolites can be produced in this way [28]. This is a relatively clean method to produce oxidation products and therefore may support metabolite identification studies. We applied electrochemical oxidation to the iodine containing SB-I and use this additional sample to illustrate the strength of hyphenating HTLC to ICP-MS. The electrochemical oxidation products were analysed in both ICP-MS and ESI-MS configuration. The total compound concentration in this sample was 5-10 times lower than in the enzymatic incubations since the optimum electrochemical conversion conditions were found to include a substrate concentration of only 10 µM (data will be published elsewhere). Figure 7 shows the ICP-MS trace of iodine and the UV chromatogram at 254 nm. Unfortunately, in ESI-MS only the substrate was found, indicating that the concentrations of the formed products were below their ESI-MS detection limits. The latter was verified using a conventional solvent gradient analysis. Nevertheless, the concentrations of the products could be determined by the HTLC-ICP-MS analysis (table 2). The electrochemical oxidation process converted more than 60% of the initial SB-I into six iodine containing products. The major product was formed at a concentration of 4 µM, while other products were formed in concentrations of 1 µM or lower.

Table 2: Retention time, peak area and determined concentration by ICP-MS of electrochemical oxidation products of SB-I.

Peak	Retention time (min)	area (counts)	concentration (µM)
1	0.5	2311	< 0.08 (0.003)
2	0.7	17516	0.39
3	1.8	9953	0.20
4	5.1	160583	4.00
5	7.7(SB-I)	151373	3.76
6	7.9	29748	0.70
7	11.7	44491	1.07
	Total	415975	10.1

In this particular case, the total concentration of all products and the parent drug was found to be the same as the total concentration of the parent drug before oxidation. This means that no dehalogenation occurred in the electrochemical oxidation and that all products detected contained iodine. If this would be the case with *in vitro* metabolism studies, mass balancing can be done which significantly contributes to the elucidation of the drug metabolism and to the metabolic and pharmacokinetic profiling. Moreover, the data in Table 2 and Figure 7 also indicate that not only the ESI-MS response but also the molar extinction coefficient may significantly change upon oxidation. For instance, peaks 4 and 5 show similar response in ICP-MS, whereas their response in UV is significantly different. Therefore, in this specific case, the assumption that conversion products have the same UV extinction coefficient cannot be made since it would significantly underestimate the concentrations of products formed by oxidation.

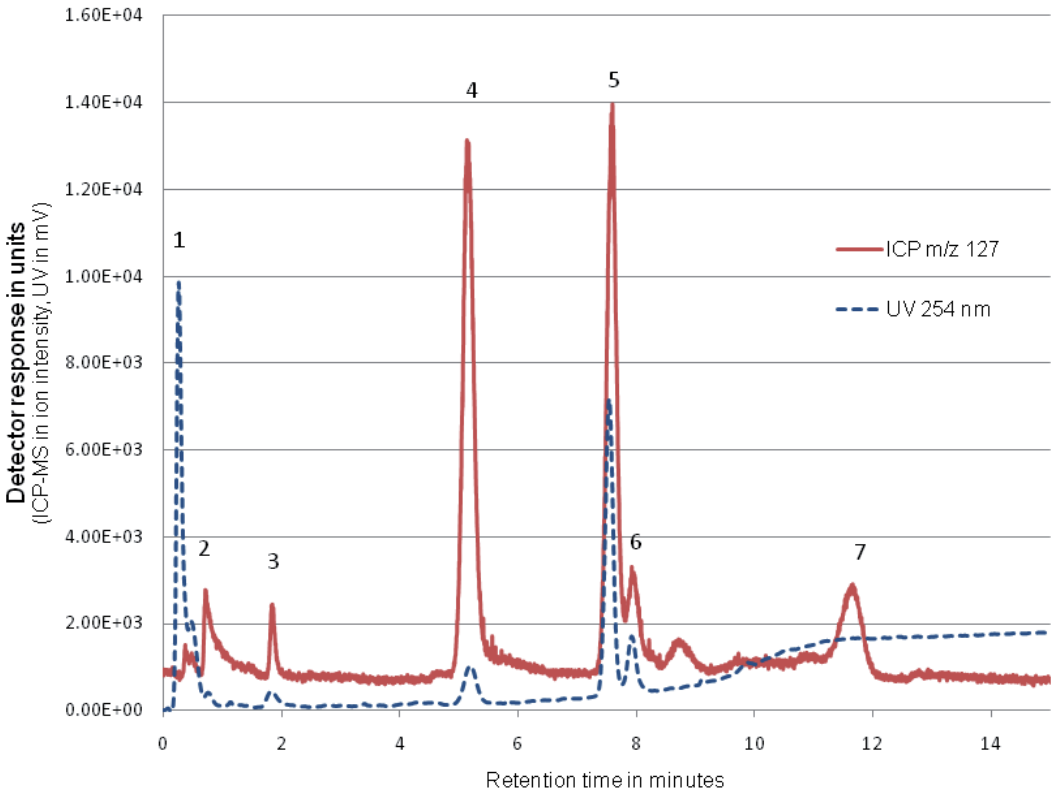

Figure 7: Overlay of HTLC-ICP-MS (solid) and HTLC-UV (dashed) chromatogram of the electrochemical oxidation products of SB-I.

4. Conclusions

The combined use of temperature gradients and isocratic solvent compositions enabled the profiling and quantification of several oxidation products of SB-I and MAPK inhibitor VIII by means of ICP-MS. Information on structures of the metabolites was obtained by ESI-HR-MS^n experiments. The detection of drug metabolites by normalized and selective detection of a halogen using ICP-MS provides valuable and complementary information on the analytes. Whenever the halogen remains incorporated within the metabolites, they are detected, can be quantified, and even mass balancing is possible. However, if oxidative dehalogenation occurs, mass balancing approaches become more difficult, especially when more than one metabolite looses the halogen from its structure.

Nevertheless, the hyphenation of HTLC to structure independent detection methods such as ICP-MS and ELSD can contribute to the toolbox of researchers for the quantification of drug metabolites and other drug related molecules, e.g. process impurities, degradation products. The importance of these kinds of quantification strategies is stressed by the observation that the difference of the position of one oxygen atom in a structure can greatly affect its response in ESI-MS and UV detection. Whether the incorporation of this atom is by P450 metabolism, electrochemical oxidation, photodegradation or any other process is irrelevant for the strategy presented in this paper.

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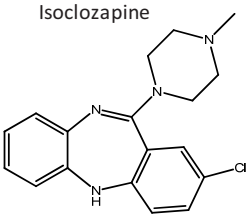
Supporting Information

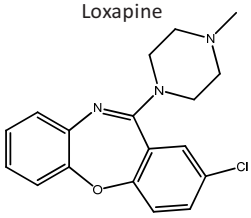
Figure S1: Structures of the compounds used in this study

Isoclozapine



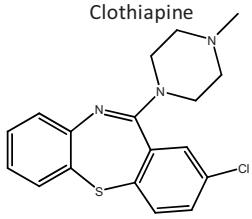
Chemical Formula: $C_{18}H_{19}ClN_4$

Loxapine



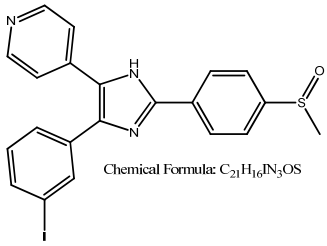
Chemical Formula: $C_{18}H_{18}ClN_3O$

Clothiapine



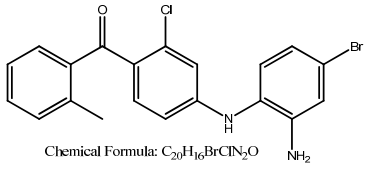
Chemical Formula: $C_{18}H_{18}ClN_3S$

SB- 203580 - Iodo



Chemical Formula: $C_{21}H_{16}IN_3OS$

P38 MAPK Inhibitor VIII



Chemical Formula: $C_{20}H_{16}BrClN_2O$

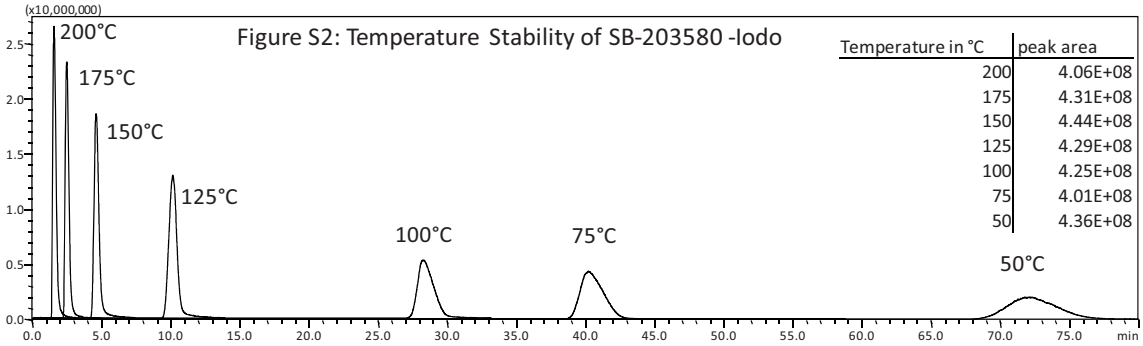
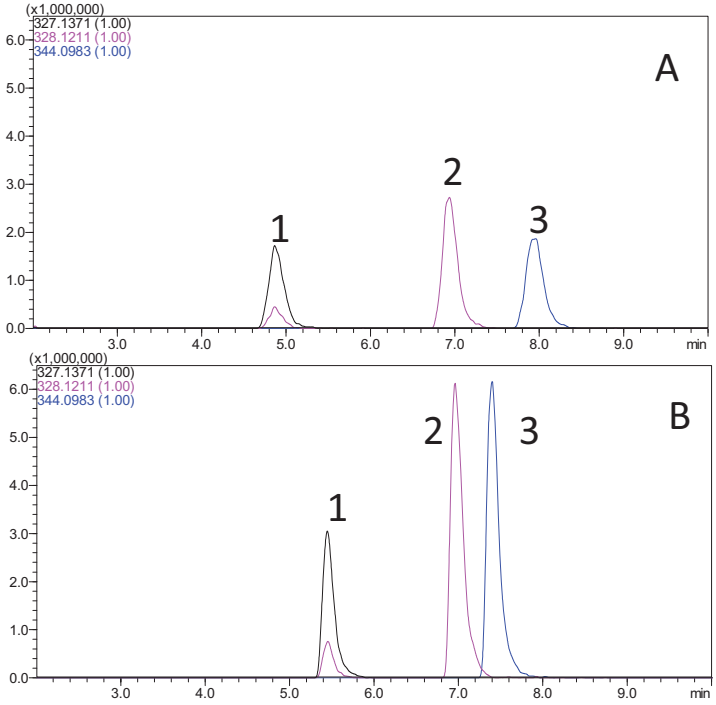


Figure S2: Temperature Stability of SB-203580 -Iodo

Temperature in °C	peak area
200	4.06E+08
175	4.31E+08
150	4.44E+08
125	4.29E+08
100	4.25E+08
75	4.01E+08
50	4.36E+08

Figure S3: Comparison of HTLC-ESI-MS and LC-ESI-MS



A: Temperature gradient with 5 % ACN

1 Isoclozapine
2 Loxapine
3 Clothiapine

B: ACN gradient isothermal 50 °C