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Development of a two-dimensional liquid chromatography system for isolation of drug metabolites

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ABSTRACT

The separation, isolation and identification of drug metabolites from complex endogenous matrices like urine, plasma and tissue extracts are challenging tasks. Metabolites are usually first identified by mass spectrometry and tentative structures proposed from product ion spectra. In many cases mass spectrometry cannot be used to determine positional isomers and metabolites have to be fractionated in microgram amounts for analysis by NMR. To overcome the difficulties associated with separation and isolation of drug metabolites from biological matrices, a new two-dimensional liquid chromatography system has been developed.

The retention times of 45 acidic, basic and neutral compounds were determined on liquid chromatographic columns with different stationary phases in order to identify two columns with highly different selectivity to be used for two-dimensional liquid chromatography.

Drug metabolites of three model compounds were first generated *in vitro* with liver microsomes and then compared with potential metabolites formed by oxidation with hydrogen peroxide catalyzed by *meso*-tetra (4-sulphonatophenyl) porphine (porphine).

The results showed that the porphine system could be used as a complementary system for the generation of phase I microsomal metabolites with high yield of some metabolites in a less complex matrix.

The two-dimensional liquid chromatography system was used to separate and isolate microsomal and porphine generated drug metabolites in off-line and on-line mode. Finally, to verify the utility of the developed system, urine samples were spiked with metabolite standards of model compounds for separation in the two-dimensional system.

Excellent separations were obtained with an amide column in the first dimension and a pentafluorophenylpropyl (PFPP) column in the second dimension. The metabolites were successfully separated from each other as well as from the complex biological matrix. The results demonstrate the applicability of the system for fractionation of drug metabolites but it could also be used in many other analytical purposes, especially for basic compounds.

Trace levels of metabolites were successfully separated in the on-line mode which failed in the off-line mode.

1. Introduction

Drugs are xenobiotics to living organisms and drugs are usually biotransformed into less toxic, less active and more hydrophilic forms to enhance their excretion from the body. The biotransformation process is generally divided into phase I and phase II reactions [1].

In phase I metabolism the parent compound is modified through hydrolysis, oxidation or reduction which increases the polarity and enhances the excretion of the compound. The most important phase I reactions of a wide range of structurally diverse xenobiotics are enzyme catalyzed oxidations performed by different isoforms of cytochrome P450 (CYP450). Phase II metabolism involves conjugation reactions that binds bulky polar groups to the substance. Phase II reactions are considered to be detoxification reactions and most often terminates the activity of the substrate and enhances elimination [2].

The metabolism of drugs can lead to unwanted products such as the formation of pharmacologically active metabolites, toxic metabolites or compounds causing drug–drug interactions. Identification and structural characterization of drug metabolites early on in the drug discovery and drug development process are important

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in order to avoid unwanted metabolic transformations. Different *in vitro* systems are often used for this purpose. Identification, structural characterization and quantification of drug metabolites play a critical role throughout the whole drug discovery and development process [3–5].

Detailed structural characterization of unknown drug metabolites or confirmation of known structures can either be performed after isolation of selected metabolites followed by NMR spectroscopy or analysis on-line by LC/MS/MS. By using a combined approach additional metabolites can be determined. Complex mixtures can be analyzed by LC/MS/MS provided that the analytes have different molecular-mass or are separated by chromatography, while NMR demands pure metabolites. The isolation of metabolites can be complex and challenging. Samples from *in vitro* studies such as sub-cellular or cellular systems, intact cells or tissue slices as well as samples from *in vivo* animal studies such as plasma, urine or tissue extracts contain a vast amount of endogenous compounds. In some cases the metabolite concentrations are extremely low, making the isolation of drug metabolites from complex biological matrices labour intensive, time consuming and technically challenging [2].

Therefore, the generation of metabolite patterns requires dedicated sample pre-treatment methods and sophisticated separation methods. Common sample pre-treatment methods such as liquid–liquid extraction, ion-pair extraction and some solid phase extraction techniques may lead to loss of metabolites and hence poor recovery. These methods could also lead to degradation of metabolites. Liquid chromatography in one dimension is often inadequate for isolation of pure metabolites for structural elucidation by NMR. The application of a two-dimensional LC approach greatly enhances the separation and facilitates the identification and characterization of low abundant metabolites [3,6].

In two-dimensional LC, samples are subjected to two separation processes with different selectivity. The total peak capacity of a two-dimensional system is equal to the product of the peak capacities in the first and second dimensions provided that the two dimensions have orthogonal selectivity, i.e. the columns separate the sample according to different retention mechanisms [7]. This high resolving power supplies a great promise for the resolution of complex biological samples [8]. The analyte should also be enriched between the two dimensions which means that the eluent from the first column should be a weak eluent on the following separation in an on-line system.

Analysis of polar, low molecular-mass analytes using LC–MS has limitations because of low chromatographic retention and low resolution. Addition of ion-pair reagents to the mobile phases to increase the retention suppresses the ionization in LC–MS analysis. Therefore, stationary phases capable of retaining polar analytes are highly desirable. The pentaflurophenylpropyl (PFPP) stationary phase exhibits both reversed and normal phase retention for polar analytes depending on the composition of the mobile phase. The PFPP phase uses multiple retention mechanisms such as ionic interactions, hydrogen bonding, dipole–dipole interactions, aromatic and π–π interactions, and hydrophobic interactions while typical alkyl phases achieve selectivity mainly through hydrophobic interactions. PFPP columns have shown increased retention especially for basic analytes. The column also works in HILIC mode, which is advantageous for analysis of highly polar compounds [9,10].

The qualitative and quantitative generation of *in vitro* metabolites depends on the experimental set-up and the model used to generate the metabolites. Structural elucidation of drug metabolites remains difficult because of the interference from biological matrices. Some chemical systems which can mimic the catalyzed oxidations performed by CYP450 has been identified [11]. Even though it is difficult to find a single chemical system which mimics all types of catalyzed reactions performed by CYP450, a combination of different chemical systems makes it possible to mimic most CYP450 catalyzed oxidations. Oxidation with hydrogen peroxide catalyzed by iron (III) *meso*-tetra (4-sulphonatophenyl) porphine (iron(III) porphine) mimics most of the reactions catalyzed by CYP450 with high yield, however the yield is low for some reactions. These chemical systems can replace complicated, time consuming classical *in vitro* methods in order to access metabolites especially during early drug development studies [11,12].

The use of a porphine system for the generation of metabolites has been investigated for several compounds [11]. In this study three model compounds were oxidized by the porphine system and compared with metabolites formed by CYP450 oxidation in microsomes.

The main goal of this study was to develop a two-dimensional LC system for the isolation of drug metabolites. For the selection of two columns with different selectivity the retention times of 45 acidic, basic and neutral compounds were determined on amide, cyano, PFPP and C8 columns, respectively. The column pair with the lowest correlation of retention times was selected for separation of metabolites. Finally, to elucidate the power of the optimized two-dimensional LC system, urine samples were spiked with standard metabolites and separated from the urine matrix.

2. Experimental

2.1. Reagents and materials

Ammonium formate, formic acid, and acetonitrile were all of analytical grade, purchased from BDH–PROLABO, Poole, England. Methanol was purchased from Fluka, Germany and hydrogen peroxide (31.3%) was from Merck, Germany. DMSO, glutathione, NADPH and potassium phosphate were from SIGMA, Germany. Iron (III) *meso*-tetra (4-sulphonatophenyl) porphine chloride was purchased from Frontier Scientific Inc., Utah, USA. Tolterodine (R)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-methylphenyl)-phenylpropanamine, BVT 2938, 1-(3-{2-[(2-ethoxy-3-pyridinyl) oxy] ethoxy}-2-pyrazinyl)-2(R)-methylpiperazine and their standard metabolites were from Pharmacia & Upjohn, while amperozide, *N*-ethyl-4-[4,4-bis (*p*-fluorophenyl) butyl]-1-piperazine carboxamide HCl and its standard metabolites were from Ferrosan, Malmö, Sweden. The structures of the model compounds are shown in Fig. 1. Rat liver microsomes were from Xeno Tech LLC, KS, USA. Water was de-ionized using a Milli-Q system (Millipore, Bredford, MA, USA). Waters-OASIS MCX and WCX SPE (solid phase extraction) cartridges 3 cm^3 (60 mg) and 6 cm^3 (500 mg) purchased from Waters, USA. Micro-spin cellulose filters (24133) were purchased from Alltech Assoc. Inc., IL, USA while 96-well plates were purchased from Costar®, Corning Inc., Corning, NY, USA.

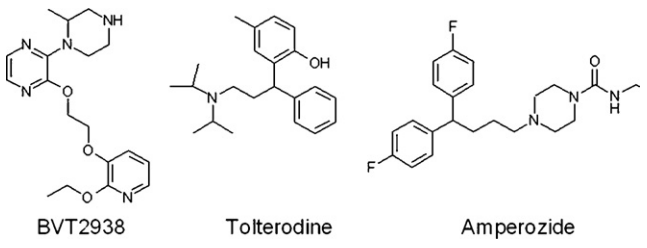

Fig. 1. Structures of BVT 2938 (1-(3-{2-[(2-ethoxy-3-pyridinyl) oxy]ethoxy}-2-pyrazinyl)-2(R)-methylpiperazine), amperozide (*N*-ethyl-4-[4,4-bis(*p*-fluorophenyl) butyl]-1-piperazine carboxamide) and tolterodine ((R)-*N*,*N*-diisopropyl-3-(2-hydroxy-5-methylphenyl)– phenylpropanamine).

2.2. Methods

2.2.1. Generation of metabolites from microsomal incubations

Microsomal incubations for the generation of phase l metabolites were performed at 37 °C with 1 μg/μl of microsomal protein (rat liver microsomes), 1 mM NADPH and 5 mM glutathione in a total volume of 1 ml 0.1 M KPO_4 buffer (pH 7.4). 10 mM stock solution of the compound was prepared in DMSO and fresh solution was added to the incubation mixture to a final concentration of 20 μM. The final concentration of DMSO did not exceed 0.5% of the total volume. As a control, rat liver microsomes were incubated without test compound. After 1 h, the incubation was terminated with one volume of ice-cold acetonitrile. Precipitated microsomal proteins were removed by centrifugation at 3500 rpm for 15 min at 4 °C. The acetonitrile used to terminate the reaction was evaporated under nitrogen and the sample was filtered through 0.45 μm micro-spin cellulose filters before subsequent analysis.

2.2.2. Generation of metabolites by porphine catalyzed hydrogen peroxide oxidation

50 μl of a 10 mM stock solution of the test compound, 35 μl of acetonitrile, 315 μl of 100 mM formic acid, 50 μl of 10 mM iron (III) *meso*-tetra (4-sulphonatophenyl) porphine chloride, and 50 μl hydrogen peroxide was incubated for 2 h at 37 °C, with addition of 50 μl of hydrogen peroxide every 30 min.

Control experiments were carried out without adding the test compounds. After the incubation the samples were frozen at −18 °C until analysis by LC/MS. For all microsomal and porphine incubations, the base peak intensity chromatograms of the expected metabolites from incubated samples and their corresponding control samples were compared in order to identify metabolites.

2.2.3. Solid phase extraction of metabolites

The samples were extracted using Waters-Oasis MCX (Mixed mode Cation-eXchange) and WCX (Mixed mode Weak Cation-eXchange) SPE cartridges.

SPE was performed with a Supelco SPE work station. Samples were diluted 1:1 with 4% ammonium hydroxide prior to extraction. Cartridges with 60 mg stationary phase were conditioned with 3 ml of methanol followed by 3 ml of water. The samples (3 ml) were then applied to the cartridges. The first washing step was done with 2 ml 2% formic acid in water for MCX cartridges and 5% ammonium hydroxide in water for WCX cartridges. The second washing for both cartridges was done with 2 ml methanol. Elution of the analytes was done with 2 ml 5% ammonium hydroxide in methanol for MCX cartridges and 2 ml 2% formic acid in methanol for WCX cartridges. The extracts were evaporated under nitrogen and reconstituted in 1 ml water containing 10% methanol.

2.2.4. LC/MS

A Shimadzu pump model LC10AD (Kyoto, Japan) was used for the LC separations. Mobile phase A consisted of 2% acetonitrile in 15 mM $HCOONH_4$ with pH adjusted to 3.6 and mobile phase B 3 mM $HCOONH_4$ in acetonitrile containing 2% water.

All the LC/MS and LC/MS/MS analyses were performed using a Sciex 2000 QTRAP mass spectrometer (Sciex, Toronto, Canada) equipped with a TurboSpray interface operated either in positive mode or negative mode. Nitrogen was used as collision gas for collision induced dissociation (CID). Reconstructed ion current (RIC) chromatograms were prepared for identification of ions using enhanced MS data. The enhanced product ion spectra (ion trap mode) were used for the identification and confirmation of substances and metabolites. The collision energy was ramped between 5 eV and 30 eV or 10 eV and 50 eV. The Analyst software (Applied Biosystems, version 1.4) was used for the instrument control, acquisition and evaluation of data.

Positive ion mode was used for the analysis of all basic compounds, all neutral compounds and for some of the acidic compounds while negative ion mode was used for most of the acidic compounds.

2.2.4.1. Evaluation of different stationary phases. All stock solutions from selected acidic, basic and neutral compounds were prepared by dissolving them in DMSO to a concentration of 10 mM. The samples were further diluted to get a final concentration of 10 μM, 20 μM or 50 μM, depending on the limit of detection (LOD) of the compounds. Samples were diluted in 2% methanol for the C8 and PFPP columns, in mobile phase A for the cyano and amide columns and in mobile phase B for the HILIC-PFPP column. Retention times were determined after injection of 30 μl standard mixture with the use of RIC chromatograms from the following columns: C8 column (10 cm × 2.1 mm, 3 μm), PFPP column (HSF 5, 10 cm × 2.1 mm, 3 μm), amide column (15 cm × 2.1 mm, 5 μm) were from Supelco, Bellafonte, PA, USA and Cyano column (Zorbax SB-CN, 5 cm × 2.1 mm, 5 μm) was from Agilent, CA, USA.

The column flow rate was set to 0.2 ml/min. In reversed phase mode, the solvent gradient conditions changed linearly from 0% to 90% B during 25 min. In HILIC mode the gradient conditions changed linearly from100% to 50% B during 25 min.

2.2.4.2. Identification and confirmation of generated metabolites. BVT 2938 and amperozide metabolites were generated by liver microsomes and by the porphine system. Tolterodine metabolites were generated by the porphine system. Separations were done using an amide column (15 cm × 4.6 mm, 5 μm) with a total flow rate of 1 ml/min and the same reversed phase gradient elution as described above. RIC chromatograms of the metabolites were used for the identification of BVT 2938 metabolites. Fragmentation pathways for BVT 2938 were examined for the metabolites generated by microsomal incubations and compared with the literature data [5]. The product ion spectra of metabolites generated in liver microsomes were compared with spectra from potential metabolites formed in the porphine system.

Generated tolterodine and amperozide metabolites were compared with the literature data for identification [13,14]. RIC chromatograms and the fragmentation pathways of standard tolterodine and amperozide metabolites were compared with the microsome or porphine generated metabolites.

2.2.4.3. Off-line two-dimensional LC separation. Metabolite samples (30 μl) were injected and separated on an amide column in the first dimension using the reversed mode gradient at 1 ml/min flow rate.

A post-column splitter (20:1) from Analytical Science Instruments, El Sorbante, CA, USA was used to split the column effluent and the high flow was collected in 7 s fractions by a Foxy JR fraction collector from ISCO, Columbus, Ohio, USA into two 96-well plates. The low flow was diverted to a Sciex 2000 QTRAP mass spectrometer for MS analysis. The peaks containing potential metabolites were pooled according to their retention times defined by the MS analysis.

The pooled fractions were evaporated under nitrogen, reconstituted in water or slightly acidified water with ammonium formate and re-injected on to the PFPP column for separation in the second dimension. A post-column splitter (20:1) was used to split the column effluent. Potential metabolites were collected according to the retention times of RIC chromatograms and the collected fractions were evaporated and confirmed using RIC chromatograms and product ion spectra using a collision energy ramp of 10–40 eV. A C8 column was used to check the purity of collected metabolites.

The mobile phase used for the separation of tolterodine and amperozide metabolites in the first dimension was the same as for the BVT 2938 metabolites. For the separation in the second dimen-

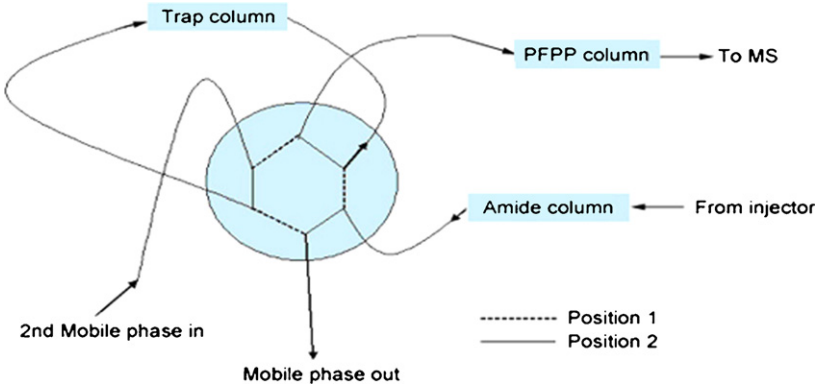

Fig. 2. Sketch of the coupled-column system for two-dimensional on-line separation. Amide column, PFPP trap column and PFPP analytical column connected to the six-port two position switching valve.

sion mobile phase B was increased linearly from 10% to 95% within 20 min.

The column flow rate for all separations in the second dimension was 1 ml/min and columns with 4.6 mm i.d. was used.

2.2.4.4. On-line two-dimensional LC separation. The samples were first separated on an amide column at isocratic conditions and the mobile phase was adjusted to achieve retention times between 4 min and 10 min. Metabolite samples were first analyzed with the amide column connected to the mass spectrometer for determination of retention times of the metabolites and time windows for column switching. In the subsequent experiment, the determined fractions of interest were trapped on a PFPP enrichment column (10 mm × 2.1 mm × 3 μm), mounted in a Cheminert six-port valve from Valco Instruments as shown in Fig. 2. After trapping the valve was returned and the analytes transferred to the second analytical column by back flushing with the same gradients for separation of BVT 2938, tolterodine and amperozide metabolites as described above. The flow rate was 0.20 ml/min for both columns with 2.1 mm i.d.

Break through volumes on the PFPP enrichment column were determined by chromatography after injection of standard compounds and measurements of the retention times and the beginning of the eluting peaks. The break through volumes were 1 ml for BVT 2938 with 25% of mobile phase B. The break through volumes with 35% B were 3.6 ml and 4.4 ml for amperozide and tolterodine, respectively.

3. Results and discussion

3.1. Evaluation of different stationary phases

The selected neutral, acidic and basic compounds were analyzed on C8, amide, cyano and PFPP columns. The retention was enhanced, especially for basic compounds on the PFPP column compared to the other columns as shown in Table 1. The PFPP stationary phase exploits different retention mechanisms which caused extended retention of basic compounds. The differences in selectivity of the PFPP column compared to C8, amide and cyano columns make it suitable for combination with one of the other columns in a two-dimensional LC system.

The correlation coefficients for the retention times between the columns showed selectivity differences between columns (Table 2). The lowest correlation coefficient was obtained for the PFPP and amide columns which indicate that these columns use different mechanisms for the retention of analytes. The retention times on the PFPP column in HILIC mode was omitted in Table 2 since a coupled-column system was desired. The scatter plot in Fig. 3 shows a high dispersion of the retention times on the amide and PFPP columns.

3.2. Generation of metabolites by the porphine system

BVT 2938 metabolites M5 (N^2-(3-{2-[(2-ethoxypyridin-3-yl)oxy]ethoxy}pyrazin-2-yl)propane-1,2-diamine), M6 (*N*-(3-{2-[(2-ethoxypyridin-3-yl)oxy]ethoxy}pyrazin-2-yl)ethane-1,2-diamine), M7 (3-(2-{[3-(2-methylpiperazin-1-yl)pyrazin-2-yl]oxy}-ethoxy)pyridin-2-ol), M8 (2-[(2-ethoxypyridin-3-yl)oxy]ethanol) and M14 (2-{[3-(2-methylpiperazin-1-yl)pyrazin-2-yl]oxy}ethanol) were identified after incubation with the porphine system and the yields were significantly higher compared to the microsomal system. The generation of M13 with unknown structure was moderate while the yield of mono-hydroxylated metabolites M1 (hydroxylamine), M2 (pyridine ring hydroxylation) and M3 (pyrazine ring hydroxylation) were low in the porphine incubation. Di-hydroxylated metabolites (hydroxylated M1) M9, M10 and M11 were not detected. All BVT 2938 metabolites have previously been described in detail [5].

The porphine system was preferred for the generation of *O*-dealkylated products of BVT 2938 such as M7, M8 and M14 and for the ring opened metabolites such as M5 and M6, while the microsome incubation was preferred for the generation of pyrazine,

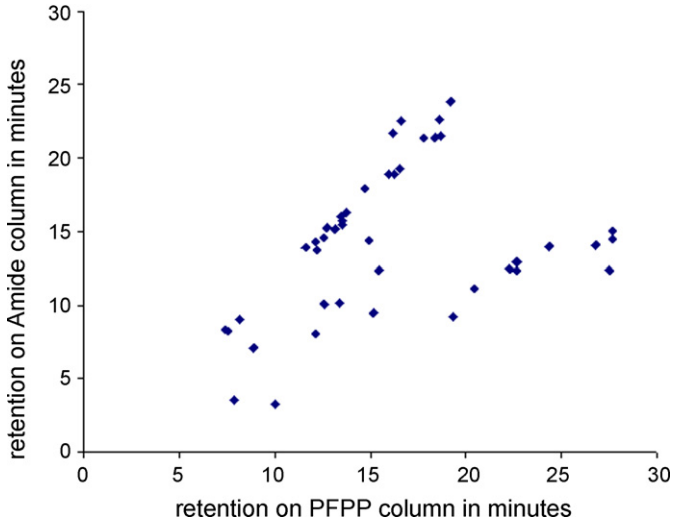

Fig. 3. Scatter plot of the retention times for the selected compounds on PFPP and amide columns.

Table 1
Retention times for selected compounds on C8, PFPP, amide and cyano columns. Retention times with asterisk were obtained in negative ion mode.

Compounds	Retention times in minutes				
Basic compounds	C8	PFPP	PFPP HILIC	Amide	Cyano
Nicotin free base	1.92	7.85	7.96	3.50	1.61
Atenolol	6.01	8.87	7.80	7.08	3.26
Alprenolol hydrochloride	12.57	22.65	8.76	12.40	9.31
Amodiaquine	7.78	19.35	8.63	9.16	7.14
Clozapine	12.78	22.66	8.01	12.90	10.44
Debrisoquin sulphate	6.73	15.15	9.88	9.49	5.43
Haloperidol	13.84	26.79	9.08	14.05	12.21
Oxybutynin chloride	15.20	27.65	7.61	14.51	12.52
Quinidine	8.86	20.44	7.84	11.13	9.58
Reserpine	15.27	27.67	7.70	15.03	14.37
2-Methyl 3-pyridil propanol	11.29	12.13	2.39	12.19	8.08
Acridine orange hydrochloride	12.99	27.50	17.89	14.40	12.37
Clotrimazole	17.83	24.31	8.12	18.13	13.99
Procainamide hydrochloride	7.13	9.98	9.17	7.45	3.29
Tamoxifen	18.84		8.69	17.31	16.36
Ticlopidine hydrochloride	12.07	22.30	8.12	14.39	12.42
Neutral compounds					
5,5-Diphenylhydantoin	14.8	13.75	1.39	16.26	10.25
7-(2-Hydroxyethyl) theophylline	7.22	7.56	1.56	8.27	4.78
Androsterone	18.56	16.15	1.62	21.67	13.23
Caffeine	6.50	8.15	1.57	8.99	5.53
Carbamarzepine	14.13	13.15	1.54	15.19	9.99
Chlorpropamide	15.76	14.69		17.88	10.06
Dexamethasone	14.37	13.55		15.76	11.21
Diazepam	17.83	16.23	1.64	18.9	12.37
Digoxin	12.55	14.91		14.37	13.27
Minoxidil	17.34	13.35	7.96	10.19	6.17
Phenylbutazone	20.79	18.37	1.44	21.42	14.52
Prednisolone	12.79	12.12	1.54	14.27	10.08
Sulphamethoxazole	12.39	12.20	1.38	13.73	7.87
Theophylline	7.06	7.42	1.58	8.31	4.54
Troglitazone	19.62	19.19		23.85	15.83
Tetrahydro cortisone	14.00	12.74		15.28	10.34
Dehydrocorticosterone	14.36	13.53		15.47	10.94
Corticosterone	14.65	13.47		16.05	11.05
Cortisone	13.23	12.58		14.58	10.06
Griseofulvin	16.56	15.47	1.43	17.45	12.32
N-Oleoyl-DL-tryptophan ethyl ester		24.56	1.63		20.24
Acidic compounds					
Deoxycholic acid sodium salt	19.64*	16.60*	1.61*	22.52*	14.14
Diclofenac sodium salt	19.62	18.62		22.65*	14.24
Flurbiprofen	19.11*	17.79*		21.35*	13.80
Indomethacin	19.44	18.68	1.80	21.47*	14.30
Ketoprofen	16.99	15.97	1.52	18.85*	11.97
Naproxen	17.10	16.54		19.30*	12.19
Phenobarbital free acid	12.56*	11.60*	1.42*	13.90*	7.28

pyridine and piperazine ring hydroxylation products such as M1, M2, M3 and the di-hydroxylated metabolites. Chromatograms recorded after oxidation of BVT 2938 with the porphine system and liver microsomes are compared in Figs. 4 and 5.

RIC chromatograms and the fragmentation patterns of metabolites M5, M6, M7, M8 and M14 showed clearly that the compounds generated by the porphine system were identical to the metabolites from the microsomal incubations. This confirms the ability of the porphine system to mimic the oxidation performed by CYP450.

The optimum conditions for maximum yield of BVT 2938 metabolites was obtained when the substance was dissolved in water, incubated at 37 °C for 2 h, with addition of 50 μl of hydrogen peroxide in every 30 min.

The porphine system was also used to generate all the tolterodine metabolites formed in liver microsomes. *N*-Dealkylated tolterodine (Ib), 5-hydroxymethyl (IIa), *N*-dealkylated 5-hydroxymehyl (IIb), and 5-carboxylic acid metabolite (IVa) were generated in large amounts while the *N*-dealkylated carboxylic acid (IVb) was generated in lower amounts. Product ion spectra of the metabolites generated by the porphine system agreed with spectra from metabolite standards.

Amperozide metabolites FG5631 (4-[4,4-bis(*p*-fluoro-phenyl)-butyl]-1-piperazine), FG5620 (deethylated amperozide), FG5657 (1-acetyl-4-[4,4-bis(*p*-fluoro-phenyl)butyl]-1-piperazine), FG-5800 (4-[4,4-bis(*p*-fluoro-phenyl)butyl]-1-piperazine-2-one) produced by incubations with rat liver microsomes and the porphine systems also gave identical product ion spectra compared to metabolites standards.

When the total ion chromatograms of the microsomal sample and porphine sample were compared, the porphine sample contained less matrix components. The metabolite yields were also high for several metabolites. Thus the porphine system could

Table 2
Correlation coefficients for retention times of all the tested compounds on C8, PFPP, cyano and amide columns.

	C8	PFPP	Cyano	Amide
C8	–	0.190	0.763	0.874
PFPP	0.190	–	0.437	0.146
Cyano	0.763	0.437	–	0.788
Amide	0.874	0.146	0.788	–

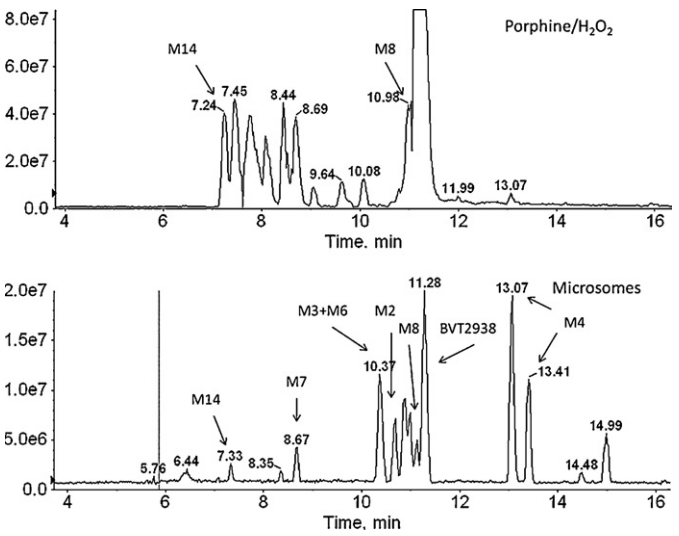

Fig. 4. Base peak intensity (180–400 Th) chromatograms recorded after oxidation of BVT 2938 with porphine/H_2O_2 (top) and rat liver microsomes (bottom) separated on the amide column with a gradient from 0% B to 90% B during 25 min.

be used as a complementary system to generate drug metabolites.

The porphine system was simple and time saving, easy to scale-up to produce metabolites needed for further testing and analysis.

3.3. Off-line two-dimensional LC separation

3.3.1. Separation of BVT 2938 metabolites generated with porphine and microsomes

Samples were extracted using mixed mode cation exchange cartridge (MCX) since extraction with mixed mode weak cation exchange cartridge (WCX) caused loss of some BVT 2938 metabolites.

The resolution was low for the separation of BVT 2938 metabolites on the amide column. The co-eluting metabolites were collected in two fractions.

Separation of those fractions on the PFPP column separated the metabolites from each other and also from the matrix components.

The purity check on the C8 column showed low purity of the metabolites produced in microsomes compared to the metabolites generated by the porphine system, due to a matrix component from the liver microsomes.

BVT 2938 metabolites (fraction 2 containing M8, M6, M5 and parent) were separated on the PFPP column in HILIC mode as the second dimension. The mobile phase gradient was from 100% B to 30% B during 25 min. In this experiment adequate separation of M6, M5 and parent was not achieved in the HILIC mode but M8 was separated with very short elution time.

Fraction 2 from the amide column was also separated on a Gemini-NX C18 (15 cm × 4.6 mm, 3 μm) column as the second dimension. The pH of the mobile phase buffer was 10.5 (10 mM NH_4HCO_3) for this analysis and all the basic analytes were in their

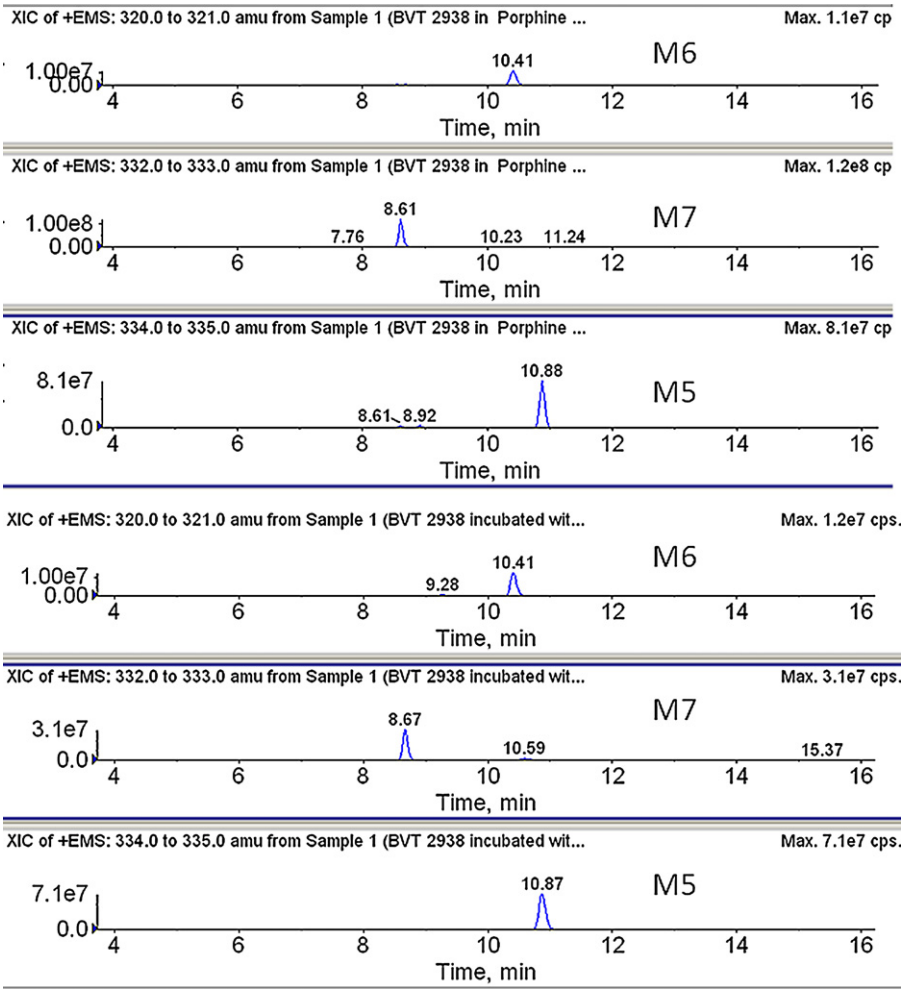

Fig. 5. Reconstructed ion current chromatograms of M5, M6 and M7 from the separations in Fig. 4 with porphine system at the top and liver microsomes at the bottom.

neutral state. Thus large difference in selectivity could be expected for basic compounds separated at acidic and basic conditions since neutral metabolites were more retained on the reversed phase column. The resolution was better compared to the separation in the PFPP-HILIC mode but insufficient for isolation and analysis. Further improvements of the gradient with the basic eluent may result in a useful system for two-dimensional chromatography but the large difference in pH may cause problems in an on-line system.

The off-line two-dimensional chromatography system had drawbacks compared to the on-line system especially during the separation of minor and trace metabolites because of adsorption losses in different parts of the equipment as well plates, vials and tubes during fraction collection. The only advantage compared to the on-line system was that all metabolites were fractionated after one injection.

3.4. On-line two-dimensional LC separation

3.4.1. BVT 2938 and its metabolites

The developed system with amide and PFPP columns was applied to analyse urine samples spiked with 10 μM and 1 μM BVT 2938 and direct injection of the urine without any solid phase extraction. BVT 2938 co-eluted with a matrix component in the first dimension that separated during two-dimensional LC. The separation of 1 μM BVT 2938 from a complex matrix like urine clearly demonstrates the separation power of the coupled system (data not shown).

The separation of BVT2938 metabolites from a microsomal incubation prepared without solid phase extraction was performed with isocratic elution with 25% of buffer B. The time windows were determined for the co-eluting metabolites and for the parent compound using RIC chromatograms. Time window 1 was set to 2.5–4.0 min (M3, M7 and M9) while time window 2 was 4.0–5.5 min(M2, M5, M6, M8 and parent) and time window 3 was 3.6–4.3 min (M2, M6, M9 and M13).

The metabolites that co-eluted during time window 1 separated well from each other and from the endogenous microsomal components in the second dimension. Product ion spectra of the metabolites were used to confirm their identity. The co-eluting metabolites M8, M2, M6, M5 and parent compound in time window 2 separated from each other as shown in Fig. 6. The two isomers of metabolite M2 were also separated from each other. Their product ion spectra confirmed them as M2. The metabolites in time window 3 were also well separated.

BVT 2938 metabolites generated by the porphine system were also analyzed on this system. The generation of mono-hydroxylated metabolites was very low in the porphine system and the metabolites were lost during off-line separation. Trace levels of mono-hydroxylated BVT2938 were successfully separated in the coupled system.

Separation of mono-hydroxylated BVT2938 demonstrates the separation ability of low and trace abundance metabolites by the on-line system. Even though the sample was not subjected to the SPE, the on-line two-dimensional separation successfully sepa-

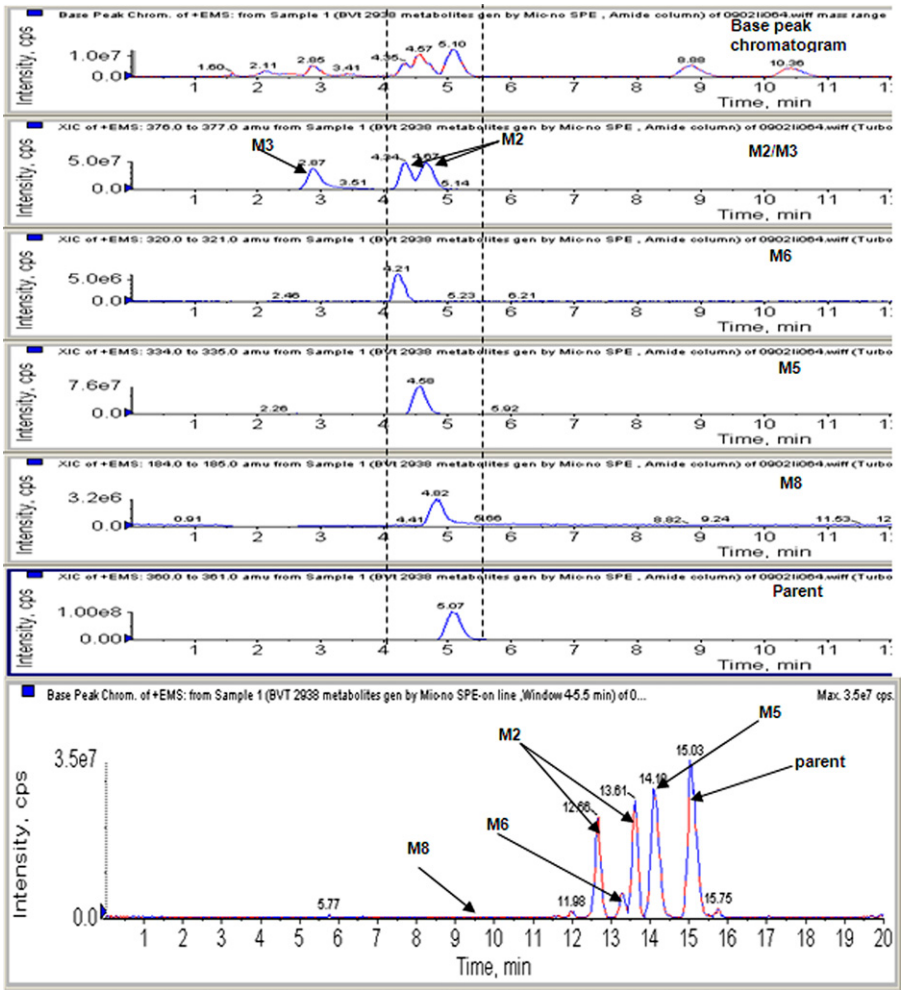

Fig. 6. Base peak chromatogram and RIC chromatograms for the separation of BVT 2938 and its metabolites M2, M3, M6, M5, M8 generated by liver microsomes in the first dimension (amide column) at the top. Base peak chromatogram for the two-dimensional separation of M2, M6, M5, M8 and parent from time window 2 at the bottom.

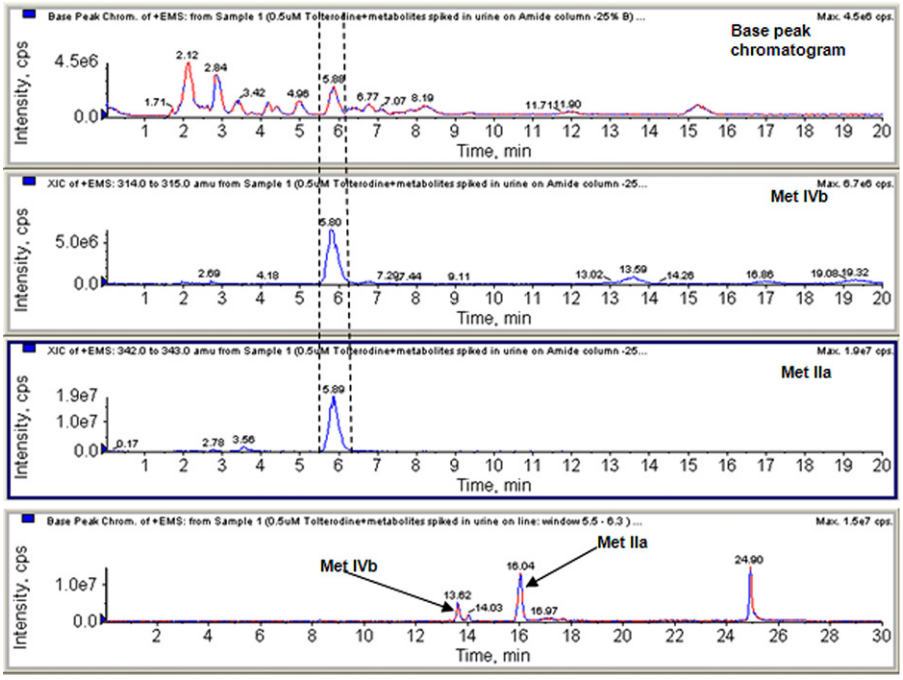

Fig. 7. Base peak chromatogram and RIC chromatograms for separation of standard tolterodine metabolites IIa and IVb in the first dimension (amide column) at the top. Base peak chromatogram for two-dimensional separations of IIa and IVb from time window 2 at the bottom.

rated low abundance metabolites of BVT 2938. This could be due to the enrichment of metabolites on the trap column that eliminated the transferring steps that caused loss of metabolites.

3.4.2. Tolterodine and its standard metabolites

Two-dimensional analysis was carried out for a urine sample spiked with 0.5 μM standard tolterodine metabolites IIa, IIb, Ib, IVb and tolterodine. The separation of metabolites in the first dimension was isocratic with 25% B. Some metabolites eluted together while some co-eluted with matrix components in the first dimension. The time windows were selected from RIC chromatograms (1: 4.0–4.5 min; 2: 5.5–6.3 min; 3: 6.7–7.5 min and 4: 14.6–16.0 min).

Tolterodine metabolite IIb co-eluted with a matrix component within time window 1 in the first dimension, was well separated from the matrix component and eluted as a single symmetrical peak in the second dimension. Metabolites IVb and IIa which co-eluted during time window 2 in the first dimension separated from each other in the second dimension as shown in Fig. 7. Metabolite IIa was well separated from an endogenous matrix component. Metabolite Ib, which co-eluted with an endogenous component within the

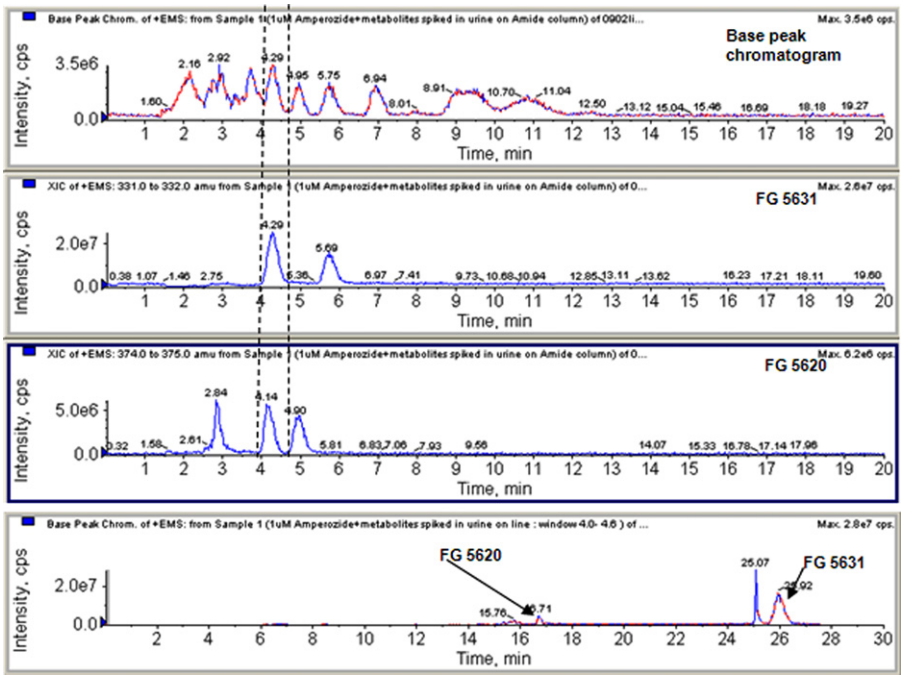

Fig. 8. Base peak chromatogram and RIC chromatograms for the separation of standard amperozide metabolites FG 5631 and FG 5620 in the first dimension (amide column) at the top. Base peak chromatogram for the two-dimensional separations of FG 5631 and FG 5620 from time window 1 at the bottom.

time window 4, separated clearly in the second dimension (data not shown).

3.4.3. Amperozide and its standard metabolites

On-line two-dimensional analysis was carried out for a urine sample spiked with 1 μM standard amperozide and its metabolites. Separation of the metabolites in the first dimension was performed at isocratic conditions with 35% B.

Amperozide metabolites FG5631 and FG5620 co-eluted in time window 1 in the first dimension. They were well separated from each other and the matrix components after separation in two-dimensions, as shown in Fig. 8. Metabolite FG 5657 co-eluted with matrix components during time window 2 and metabolite FG 5800 co-eluted with matrix components during time window 4 in the first dimension also clearly separated from matrix components during two-dimensional separation. Amperozide also co-eluted with matrix component during time window 3 in the first dimension but separated in the second dimension.

The results discussed above have demonstrated the separation power of the two-dimensional liquid chromatography system for the separation of metabolites from each other and also from complex biological matrices. The successful results were due to a high degree of orthogonality of retention times for the separation on the amide and PFPP columns. Other advantages of the on-line system was the reduction of analysis time and simplicity to automate.

4. Conclusions

The low correlation of retention times between the amide and PFPP columns indicated large differences in the selectivity between the columns. Hence the combination of these two columns was employed successfully for two-dimensional liquid chromatography.

The two-dimensional system was highly applicable for separation and isolation of basic metabolites from biological matrices. Unresolved peaks in the first dimension were well separated in the second dimension. In this work we studied three drugs and their metabolites but the system described may have a wide applicability for trace analysis of single analytes in complex matrices after minor adjustment of the chromatographic conditions. The high separation power of the two-dimensional system could be useful if less selective detectors are used instead of mass spectrometry. Chemical oxidation with the porphine system was useful to mimic phase I oxidations performed by CYP450. The porphine system gave considerably higher yields for ring opened and *O*-dealkylated products of BVT 2938 compared to liver microsomes. The yields were low for mono-hydroxylated products and di-hydroxylated products of BVT 2938 were not detected at all.

The purity of metabolites produced by the porphine system was higher compared to metabolites from liver microsomes. The utility of the developed system for fractionation and analysis by NMR remains to be demonstrated.

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