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o-(Pentafluorobenzyloxycarbonyl)benzoyl chloride: a novel electrophoric derivatisation reagent for amino compounds designed for negative ion chemical ionisation mass spectrometry

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The synthesis of a novel electrophoric derivatisation reagent, *o*-(pentafluorobenzyloxycarbonyl)benzoyl chloride, is described. The reagent was tested against selected primary and secondary amino compounds as analytical targets. The derivatives exhibit excellent mass spectral properties under negative ion chemical ionisation (NICI), i.e. reduced fragmentation and thus high ion current for the targeted *m/z* during analysis. Since the reagent bears a pentafluorobenzyl ester group, resulting mass NICI mass spectra were expectedly dominated by dissociative resonance electron capture typically observed with these compounds. The reagent is suitable for detecting volatile primary and secondary amines with high sensitivity. Background is reduced by a shift in detected *m/z* and retention time, as demonstrated for the analysis of the drug methylphenidate from human plasma. Copyright © 2010 John Wiley & Sons, Ltd.

In quantitative analysis of trace amounts there is a never-ending search for ultimate sensitivity and selectivity. Analytical assays based on mass spectrometry have emerged as reference methods due to the well-known inherited characteristics, allowing the use of stable isotope labeled internal standards and the choice of various ionisation and detection modes. In particular, negative ion chemical ionisation (NICI) provides an extremely sensitive tool in gas chromatography/mass spectrometry (GC/MS). Basically, using this detection mode, there are two ways to produce negative ions from an uncharged molecule: by resonance electron capture (REC) and dissociative resonance electron capture.[1] REC produces a molecular radical anion in its excited state that must be stabilised to prevent electron autodetachment, whereas dissociative REC results in non-radical anions with a distribution of internal energies, depending largely on the leaving group and its ability to absorb excess internal energy, which increases with its size.[2–4] Besides mass-selective detection, the REC process adds another dimension of selectivity to the analytical process. REC is strongly dependent on the electron affinity of the target molecule, and thus on its electronegativity, rendering it as electrophoric. As many analytical targets possess only low electron affinity, chemical derivatisation is usually employed to enhance NICI response. There are many reagents available, the most widely used compounds are perfluoroacyl[5–8] and pentafluorobenzyl[9–11] (PFB) derivatives. For perfluoroacylates, REC response is usually high, but, interestingly, the molecular radical anion is seen in relative low abundance in many cases. Instead of this, sequential loss of hydrogen fluoride (HF) from the molecular anion is observed, sometimes displaying a complex fragmentation pattern in the NICI mass spectra and hence reducing the ion current of the 'diagnostic' fragment chosen for quantification.[12,13] Thus, derivatives yielding low fragmentation under NICI with ions in the higher mass range are ideally suited for enhancement of sensitivity. This is met by PFB derivatives of carboxylic acids and phenols, where both, the carboxylate or phenolate anion and PFB radical formed by dissociative REC, are stabilised by resonance and hence produce virtually only these ions without any further fragmentation in many cases.[1,14] Up to now, these extremely sensitive derivatives are only available for carboxylates and phenolates, using the PFB bromide as a reagent. It was thus the aim of this study to develop a new reagent that produces derivatives of amino compounds with a NICI response similar or identical to the PFB derivatives of carboxylic acids or phenols.

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EXPERIMENTAL

Chemicals and reagents

Pentafluorobenzyl alcohol was purchased from ABCR (Kahrlsruhe, Germany). Phthalic anhydride, heptafluorobutyric anhydride, memantine.HCl, amantadine.HCl, metamphetamine.HCl and amphetamine sulfate were supplied by Sigma-Aldrich (Vienna, Austria). [$^{18}O^{2}H_{3}$]-methylphenidate was self-synthesised as previously desribed.[12] Silylation grade pyridine was obtained through Pierce (Rockford, IL,

USA). Racemic (±)-*threo*-methylphenidate hydrochloride was from Medice (Iserlohn, Germany). All other substances, solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

Synthesis of *o*-(pentafluorobenzyloxycarbonyl)benzoyl chloride (PBBCl)

In a screw-capped reaction vial phthalic anhydride (148 mg, 1 mmol) and pentafluorobenzyl alcohol (PFB-OH, 198 mg, 1 mmol) were dissolved in benzene (300 μL) and pyridine (100 μL) was added. The mixture was kept at 100°C for 2 h. After acidification with concentrated HCl the intermediate product was extracted with chloroform. The organic phase was dried over anhydrous sodium sulfate and concentrated under nitrogen to yield an oily residue that solidified on standing in over 90% yield. The crude compound was recrystallised from benzene or chloroform to yield the pure mono-PFB ester of phthalic acid as a white solid, as judged by straight-phase HPLC on silica (20% ether in n-hexane as a mobile phase) and GC/MS after conversion into the trimethylsilyl (TMS) ester (MS (EI): *m/z* 418 ($M^{\bullet+}$), *m/z* 403 ($M^{\bullet+}$–$^{\bullet}CH_3$), *m/z* 329 ($M^{\bullet+}$–TMSO$^{\bullet}$), *m/z* 181 ($C_7H_2F_5^+$)

Each of the above intermediate products (36.4 mg) were treated with thionyl chloride (0.5 mL) at room temperature for 1 h. Excess reagent was removed under nitrogen and the oily residue dissolved in dichloromethane (10 mL), yielding a 10 mM solution of the PBBCl reagent.

Derivatisation with PBBCl

Aqueous solutions of amphetamine sulfate, metamphetamine.HCl methylphenidate.HCl, memantine.HCl and amantadine.HCl) were made alkaline with carbonate buffer (pH 9.0) and extracted with n-hexane. The solvent was removed under nitrogen and the residue was treated with 100 μL of reagent solution (PBBCl, 1 mM in dichloromethane) for 30 min at room temperature. Solvent was removed under nitrogen and the residue reconstituted in 100 μL ethyl acetate and 1 μL was subjected to analysis by GC/NICI-MS. Alternatively, the following procedure can be used: The dry residue after hexane extraction was treated with 200 μL of carbonate buffer and 200 μL of reagent solution. The mixture was shaken for 30 min at room temperature. Thereafter, 1 mL of n-hexane was added, the mixture vortexed and the upper hexane phase collected. Concentration and reconstitution was carried out as described above.

For assessment of reaction yields, 20 ng of methylphenidate was treated with PBBCl as described above. After certain time points (2-10-15-20-30 min) 50 μL of heptafluorobutyric anhydride (containing 2% heptafluorobutyric acid) were added and the mixture left for an additional 30 min. A 50:50 response was estimated by derivatising equal amounts separately (as HFB and PBB derivatives) and analysing the combined solutions.

Gas chromatography/mass spectrometry

A Finnigan TRACE 8000 gas chromatograph coupled to a Finnigan TRACE quadrupole mass spectrometer (ThermoQuest, Vienna) was used. The gas chromatograph was fitted with a BPX5 fused-silica capillary column (30 m × 0.25 mm i.d., SGE). The injector was operated in the splitless mode at 280°C. Helium was used as a carrier gas at a constant flow rate of 2.0 mL/min. The initial column temperature was 160°C for 1 min, followed by an increase of 40°C/min to 315°C and an isothermal hold of 10 min. For analysis of heptafluorobutyrates, the initial column temperature was set to 80°C. The mass spectrometer transfer line was kept at 320°C. NICI was performed with methane as a moderating gas at an electron energy of 70 eV and an emission current of 0.250 A.

Extraction and derivatisation of methylphenidate

Methylphenidate was extracted from spiked plasma as previously described.[12] Briefly, 1 mL of plasma was spiked with 0.5 ng of [$^{18}O^2H_3$]-methylphenidate internal standard and different concentrations of methylphenidate (4.5-9-18-36-72 pg). Plasma was made alkaline by addition of carbonate buffer (pH 9.0) and extracted with 2.5 mL of n-hexane for 15 min. After centrifugation, the organic layer was brought to dryness under nitrogen and derivatised as described above. The residue was reconstituted in 60 μL ethyl acetate and 2 μL were subjected to GC/NICI-MS analysis.

RESULTS AND DISCUSSION

PBBCl reagent preparation and derivatisation

The use of a bifunctional carboxylic acid as starting compound for the synthesis of an electrophoric derivatisation reagent provided the functionalities necessary for the preparation of a typical acylation agent suitable for amino compounds, as shown in Fig. 1. Alcoholysis of phthalic anhydride with PFB-OH proceeded smoothly to yield the mono-PFB ester of phthalic acid. Subsequent formation of the acyl chloride occurred quantitatively to provide the reactive group for acylation reactions. As the intermediate acid product is solid it can be conveniently purified by crystallisation. The acyl chloride reagent can be prepared freshly on demand, although we have used reagent solutions over several weeks without any noticeable deterioration when stored in a freezer.

We have used the general procedure described herein to prepare the PBB derivatives of various primary and secondary amines without optimising reaction conditions for each substance. The derivative formed smoothly with the compounds tested by simply adding reagent solution at room temperature. Some primary amines could be derivatised by simply adding reagent solution to the alkaline hexane extract. This is of particular advantage for volatile amines that would suffer from considerable loss during solvent evaporation. We have performed derivatisation reactions with and without diisopropylethylamine as an acid scavenger and found no enhancement of derivative yield. With methylphenidate as an example we have established reaction yields under these conditions. Thus, reaction has already progressed to 78% after 2 min, gradually increasing to >98% after 30 min. The PPB derivatives showed good stability. Derivatised samples were stored under

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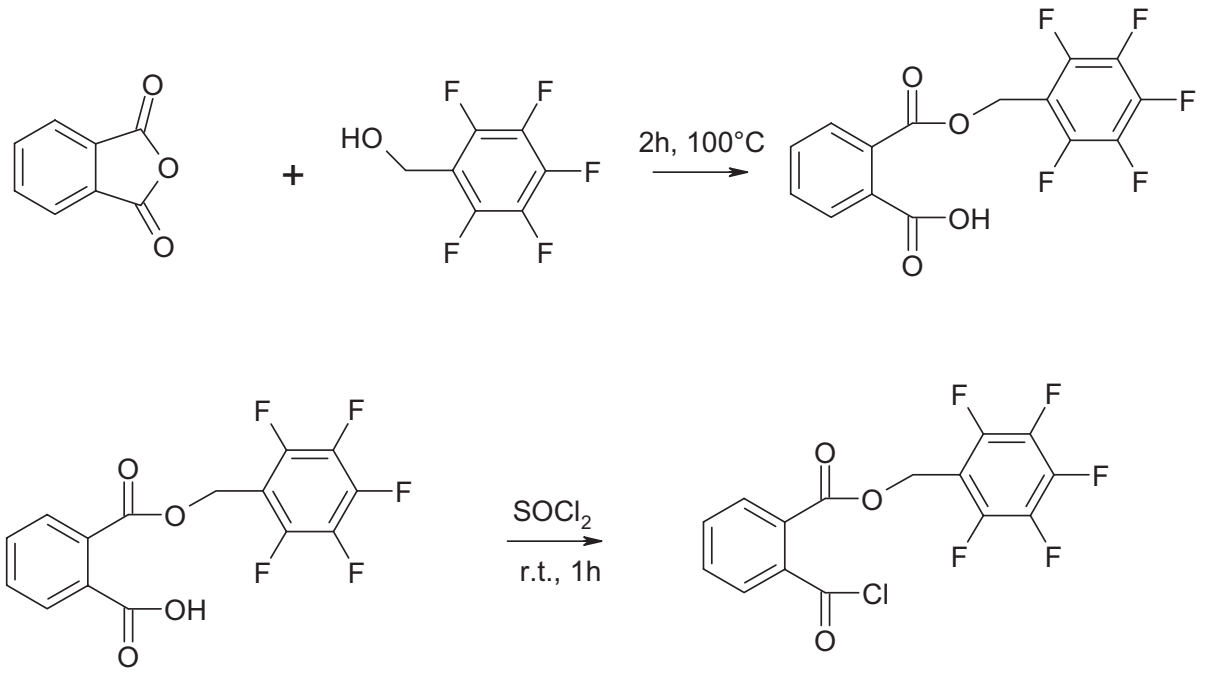

Figure 1. Reaction scheme for the preparation of the PBBCl reagent.

various conditions and re-analysed. There was no decrease in signal intensity when samples were analysed after storage for 2 weeks at room temperature and 4 weeks at –20°C. As the introduced functional group is large, volatility of the PBB derivatives is reduced. The reagent is thus well suited for the analysis of amino compounds of low to moderate volatility. Under the conditions employed no reaction was observed with alcoholic and phenolic hydroxyls. Using harder conditions (high temperatures, base catalyst), there may be some reaction with hydroxyl groups, but we did not see any reaction under the mild conditions described here for phenol, 2,3-dichlorophenol, pentachlorophenol, stearyl alcohol, and butanol. Thus the range of possible interference during analysis is reduced to amino compounds.

NICI-MS of PBB derivatives

NICI as a soft ionisation technique can be expected to show reduced fragmentation and more high-mass ions as compared to EI. This is, however, only true to a certain extent when for example perfluoroacyl derivatives are measured. These derivatives produce molecular anions by REC, and besides delivering fragment ions in the higher *m/z* range up to the molecular ion, fragmentation is quite intense which results predominantly from the sequential loss of HF from the molecular anion. We have observed this fragmentation pattern frequently after derivatisation with heptafluorobutyric anhydride,[12,13] thus suggesting the fluoroacyl part to mainly direct this pathway. In Fig. 2 the NICI mass spectra of methylphenidate heptafluorobutyrate and amphetamine pentafluorobenzoylate are shown. Fragmentation is intense in both cases, the molecular radical anion is not (amphetamine) or only weakly (methylphenidate) present. The proportion of the total ion current is very low, no matter which fragment ion is chosen for quantification.

On the other hand, fragmentation under NICI conditions can be drastically reduced to one or two fragment ions with

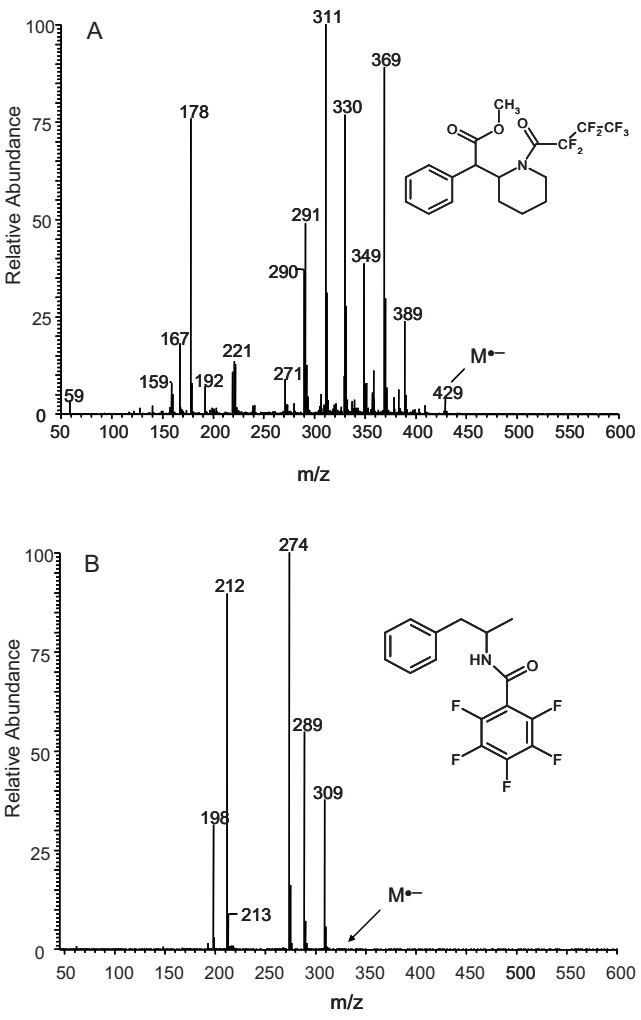

Figure 2. NICI mass spectra of methylphenidate heptafluorobutyrate (A) and amphetamine pentafluorobenzoate (B).

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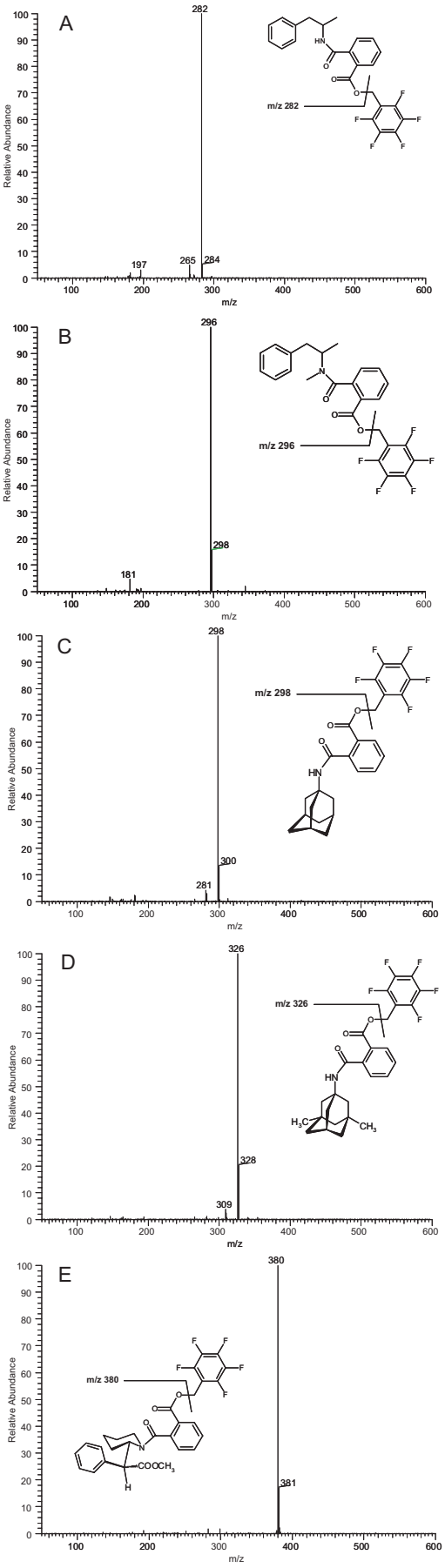

Figure 3. NICI mass spectra of PBB derivatives of amphetamine (A), metamphetamine (B), amantadine (C), memantine (D), and methylphenidate (E).

striking intensity in certain cases: PFB derivatives of carboxylic acids undergo dissociative REC when subjected to NICI that leaves the carboxylate anion with high stability and high abundance.[1,14] We have observed this unique feature also with pentafluorocarbamates and pentafluorocarbonates.[15,16] It could thus be expected that PBB derivatives would also show this fragmentation mechanism and yield similar results. The NICI mass spectra of the tested target compounds after PBB derivative formation are given in Fig. 3. In any case, the carboxylate anion is present with striking abundance. Fragmentation is extremely reduced or absent. For the PBB derivatives of memantine, amantidine and amphetamine there is a fragment ion at $[M^{\cdot -}-17]$ with low relative abundance, which is not observed in the NICI mass spectra of the secondary amines, methamphetamine and methylphenidate. It is likely that rearrangement under hydrogen transfer leads to the final neutral loss of ammonia from the primary amine derivative.

Gas chromatography of PBB derivatives

Besides their unique fragmentation behaviour PBB derivatives of amino compounds also show certain benefits in GC analysis. Due to the bulky PBB group retention times are shifted dramatically compared to heptafluorobutyrates. As the latter produce highly volatile derivatives, the retention time of certain analytes may be right within a region of high matrix interference when complex matrices such as blood plasma are encountered. In Fig. 4 analysis of a plasma extract of the drug methylphenidate is shown after derivatisation with HFBA and PBBCl. A volume of 1 mL of human plasma was spiked with 2 ng methylphenidate and 4.7 ng of the internal standard, $[^2H_3^{18}O]$-methylphenidate. HFB derivatives elute at a retention time of 5.80 min, whereas PBB derivatives are seen at 10.95 min. It is evident from this comparison that matrix interference is by far more prominent with the heptafluorobutyrates. This may additionally be due to the fact that HFBA also reacts with hydroxyl groups and thus extends the range of possible interfering compounds. As a result of reduced fragmentation, the peak area measured for the HFB derivative in a separately derivatised 1:1 mixture of HFB and PBB derivatives was only 14.7% of the PBB compound. Thus, for methylphenidate, a sensitivity gain of 7 is achieved for the GC/MS detection. The shift in retention time is even 2 min larger, as indicated in Fig. 4, since for the heptafluorobutyrates the initial GC temperature was lowered to 80°C. Our previously described method for the determination of methylphenidate using HFB derivatives had a detection limit of 72 pg/mL plasma.[12] Thus, we have established a calibration curve for this compound in human plasma after PBB derivatisation at concentrations below that limit. For the concentration range investigated (4.5–72 pg/mL plasma) we have found a linear response ($r^2 = 0.9996$) with a signal-to-noise ratio of 96 at 4.5 pg/mL.

In general, sensitivity is enhanced by the PBB reagent described here in comparison to common fluoroacylates. Additionally, the shift in retention time provides a region of lower interference from the matrix. Thirdly, the selectivity of the reagent for amino compounds also reduces interference from biological matrices.

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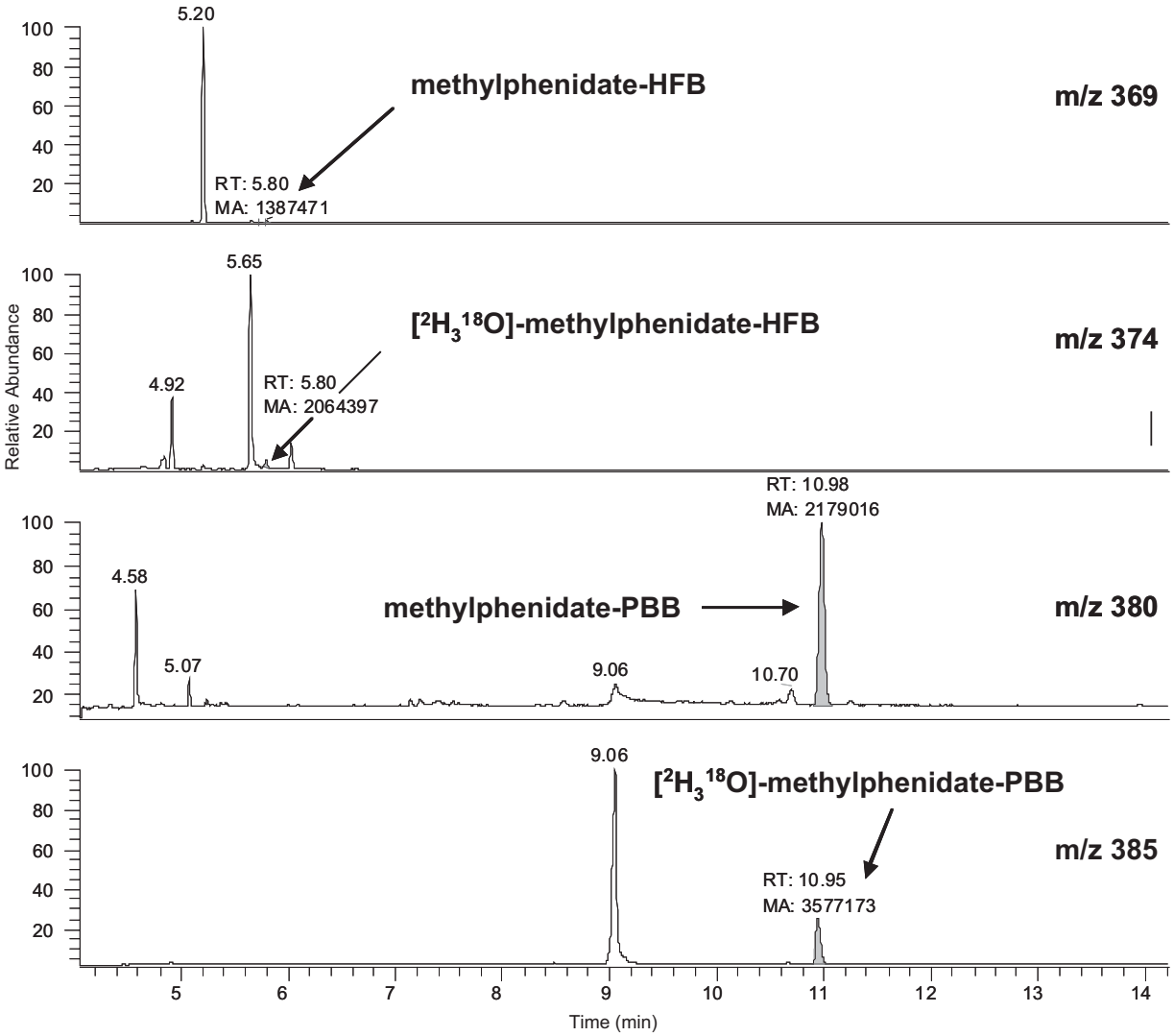

Figure 4. Chromatograms obtained after analysis of human plasma spiked with 2.0 ng/mL of methylphenidate and 4.7 ng/mL of the stable isotope labeled internal standard. Identical samples were converted into the PBB and HFB derivatives, respectively, and analysed by GC/NICI-MS.

CONCLUSIONS

In this study we report the synthesis of a new derivatisation reagent, PBBCl. The compound is reactive towards primary and secondary amines and displays ideal characteristics for analysis by GC/NICI-MS. NICI response is better than with the established heptafluorobutyryl derivatives. The unique NICI mass spectra obtained allow specific detection. The reagent can be prepared freshly on demand by a simple procedure. The PBB derivatives are sufficiently stable, as repeated injections of one prepared sample on several consecutive days did not show any loss of signal intensity. The reagent described herein allows the beneficial PFB derivatisation hitherto restricted to phenolates and carboxylic acids also to be extended to amino compounds, making them amenable to highly sensitive assays.

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